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Valorization of olive pomace through combination of biocatalysis with supercritical fluid technology

Dissertação para obtenção do Grau de Mestre em Biotecnologia

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Resumo

Implementou-se um método de extracção de óleo a partir de bagaço de azeitona por meio de dióxido de carbono supercrítico ($scCO_2$), tendo-se obtido um rendimento de 25,5 +/- 0,8% ($g_{óleo}/g_{resíduo\ seco}$). Por extracção Soxhlet com hexano obteve-se um rendimento de extracção de óleo de 28,9 +/- 0,8%, o que se traduz numa eficiência de 88,4 +/- 4,8% para o método supercrítico. O método de extracção por $scCO_2$ foi optimizado para as condições de 50 MPa e 348,15 K, tendo-se calculado um *loading* do óleo de 32,60 g oil/kg CO_2.

Como prova de conceito, usou-se o bagaço de azeitona como matéria-prima para a produção de biodiesel, num processo combinando o uso de lípase como catalisador com o uso de $scCO_2$ como solvente, e integrando os passos de extracção do óleo do bagaço, transesterificação do óleo em biodiesel e posterior separação deste. Nas experiências realizadas conseguiu-se atingir purezas de 90% de FAME (ésteres metílicos de ácidos gordos), com os seguintes parâmetros de operação: um rácio molar óleo:metanol de 1:24; tempos de residência de 7,33 e 11,6 minutos; pressão de 40 MPa; temperatura de 313,15 K; e Lipozyme (*Mucor miehei*; Sigma-Aldritch) como enzima. Registaram-se no entanto ao longo das experiências oscilações na pureza do biodiesel recuperado que se poderão dever a acumulação de metanol no reactor enzimático.

Finalmente, foi analisado o conteúdo fenólico do bagaço de azeitona, e o efeito dos processos de secagem – no forno ou por liofilização – e dos métodos de extracção – método hidro-alcoólico e o método supercrítico – no teor de fenóis. Verificou-se que a secagem do bagaço no forno preservou 90,1 +/- 3,6% do conteúdo fenólico total. Cerca de 62,3 +/- 5,53% do conteúdo fenólico do bagaço seco no forno foi extraído com $scCO_2$ a 60 MPa e 323,15 K. Foram identificados e quantificados sete compostos fenólicos – hidroxitirosol, tirosol, oleuropeína, quercetina, ácido cafeico, ácido ferúlico e ácido p-coumárico – por HPLC.

Palavras-Chave: biodiesel, transesterificação, dióxido de carbono supercrítico, bagaço de azeitona, óleo de bagaço de azeitona, fenóis.

Abstract

A supercritical carbon dioxide ($scCO_2$) based oil extraction method was implemented on olive pomace (alperujo), and an oil yield of 25,5 +/- 0,8% ($g_{oil}/g_{dry\ residue}$) was obtained. By Soxhlet extraction with hexane, an oil extraction yield of 28,9 +/- 0,8 % was obtained, which corresponds to an efficiency of 88,4 +/- 4,8 % for the supercritical method. The $scCO_2$ extraction process was optimized for operating conditions of 50 MPa and 348,15 K, for which an oil loading of 32,60 g oil/kg CO_2 was calculated.

As a proof of concept, olive pomace was used as feedstock for biodiesel production, in a process combining the use of lipase as a catalyst with the use of $scCO_2$ as a solvent, and integrating the steps of oil extraction, oil to biodiesel transesterification and subsequent separation of the latter. In the conducted experiments, FAME (fatty acid methyl ester) purities of 90% were obtained, with the following operating parameters: an oil:methanol molar ratio of 1:24; a residence time of 7,33 and 11,6 mins; a pressure of 40 MPa; a temperature of 313,15 K; and Lipozyme (*Mucor miehei*; Sigma-Aldritch) as an enzyme. However, oscillations of FAME purity were registered throughout the experiments, which could possibly be due to methanol accumulation in the enzymatic reactor.

Finally, the phenolic content of olive pomace, and the effect of the drying process – oven or freeze-drying – and the extraction methods – hydro-alcoholic method and supercritical method – on the phenolic content were analysed. It was verified that the oven-drying process on the olive pomace preserved 90,1 +/- 3,6 % of the total phenolic content. About 62,3 +/- 5,53% of the oven-dried pomace phenolic content was extracted using $scCO_2$ at 60 MPa and 323,15 K. Seven individual phenols – hydroxytyrosol, tyrosol, oleuropein, quercetin, caffeic acid, ferulic acid and p-coumaric acid – were identified and quantified by HPLC.

Keywords: biodiesel, transesterification, supercritical carbon dioxide, olive pomace, olive pomace oil, phenol

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Abbreviations and symbols

C - Celsius
µL – Microliter
BPR – Back Pressure Regulator
FAME – Fatty Acid Methyl Ester
G – Gram
HPLC – High Performance Liquid Chromatography
K – Kelvin
Kg – Kilogram
Min – Minute
mL – Millilitre
MPa – Mega Pascal
OP – Olive Pomace
OPO – Olive Pomace Oil
PTV – Programmable Temperatures Vaporizing
S – Seconds
$ScCO_2$ – Supercritical Carbon Dioxide

CHAPTER 1: STATE OF THE ART

1.1. Current and future fuel needs for the transportation sector, and environmental consequences – an overview

The transportation sector is in constant expansion, due to the increasing number of cars and other vehicles around the world. This sector is the most oil demanding sector, accounting for 55% of the global oil consumption in 2012.[1] Although oil is for now the largest source of energy, it is known that it is not renewable, and there are constant attempts to predict when it will be depleted. By the end of 2014, the proven oil reserve is equivalent to 52,5 years of current global production. [2]

Climate change is currently believed to the most pressing global environmental problem, with possibly catastrophic consequences for biodiversity and human lives. In 2012, the global CO_2 emissions were over 31000 million tons – of which around 23% (7187 million tons) correspond to the transportation sector. [3] Short-term CO_2 emission predictions indicate that each year, the global emissions will grow, perhaps to as much as 36000 million tons in 2020. [4] But longer-term (up to the year 2100) predictions for the rate of increasing carbon dioxide emissions vary, since they depend on various scenarios, including the implementation and success of several possible long-term plans against climate change. [5]

A serious dilemma thus exists: modern society needs to eventually adopt alternatives to fossil fuels due to their future exhaustion and their environmental consequences, without compromising its energy demand.

1.2. Biodiesel as an emerging energy resource

In the ongoing global effort by the scientific community to investigate possible sources of energy other, one of the most interesting alternatives is biodiesel. [6]

Biodiesel, consisting of fatty acid alkyl esters, is derived from vegetable oils or animal fats, for use in compression–ignition (diesel) engines. It is derived from triglycerides (fatty acid esterified with glycerol) existing in organic oils and not the hydrocarbons in petroleum. [7]

The growing interest in developing biodiesel as an alternative to fossil fuels comes from its interesting properties. It has an energy content and physical and chemical properties similar to conventional diesel fuel, allowing its use in diesel engines, either alone or mixed with diesel, without modifications to the engine being required. It can be stored, pumped and handled using the same infrastructure, machinery, and protocols usually employed for conventional diesel fuel. It also does not produce explosive vapors, and has a higher flash point than diesel fuel. [7]

Biodiesel is also an environmentally friendlier fuel compared to fossil fuels. It is a quasi-neutral fuel regarding CO_2, since the only emissions are those previously fixed by photosynthesis. Thus, biodiesel helps fixing the carbon dioxide balance in the atmosphere. It

also has reduced emission of important atmospheric contaminants – particles in suspension, CO, polycyclic aromatic hydrocarbons, unburned hydrocarbons and also NO_x and SO_x due to lower concentrations of these compounds. It is biodegradable and has lower health risks than fossil fuels, being just slightly toxic in soils or aquatic environments.[7]

The environmental friendliness of biodiesel can be increased by the development and optimization of more efficient processes for production and purification. Possible avenues include renewable sources of oil, use of enzymatic catalysts, and alternative substrates. [7]

Biodiesel has been in use in many countries such as United States of America, Malaysia, Indonesia, Brazil, Germany, France, and Italy. The biodiesel industry is a rapidly growing activity, with global biodiesel production increasing from 15000 barrels per day in 2000 to 430000 barrels in 2012. The European Union accounted for about 40% of the 2012 production, with Germany being the greatest producer, filling out 13% of the world's production by itself (54700 barrels). The United States of America is the greatest producer in the world, accounting for about 15% of the world's production. [8]

While increasing oil prices have been leading to biodiesel becoming viable in the medium term, locally produced biofuels are currently not feasible without any subsidy.[7] Even so, despite the effects of economic recession in biofuel development in some countries, biofuels production is expected to grow at an average of nearly 4% per year until 2030. With the European Union 2009 Renewable Energy Directive setting the target of 20% renewable energy by 2020, biodiesel is seen as part of the solution to the energy and economic challenges currently faced. [9]

While current fossil fuel reserves can be exhausted in as much as a few decades, biodiesel is a renewable resource, and because it can be produced from recycled waste oils and agricultural surpluses, biodiesel can lead to valorization of such residues while providing more employment in rural areas. This will be elaborated upon further on. [7]

1.2.1 Properties of biodiesel

In general, biodiesel's benefits and drawbacks compared to petroleum-based diesel fuel are[6]:

Table 1.1 – Summary of biodiesel's advantages and disadvantages compared to diesel fuel

Advantages	Disadvantages
• Renewable and readily available • Non-toxic and biodegradable • Helps rural development • Allows valorization of waste products • Production is less time-consuming • No need for drilling • Any country can produce it at a local level • Safer to transport, handle and storage • Good combustion characteristics • Less smoke and carbon dioxide emissions • Less ignition delay • Better lubricant properties • Does not usually require engine modifications	• High production price • Waste disposal and washing water problems • Lower energy content - higher fuel consumption. • Lower thermal efficiency • Worse low-temperature properties • Greater formation of deposits • More viscous • More prone to oxidation • Combustion produces higher NOx amounts and also some oxygenated hydrocarbons • Can cause corrosion in vehicle material, and engine durability problems

In terms of resultant engine performance when applied to a diesel engine, biodiesel can be characterized using a number of parameters, directly related to its physical and chemical properties. Different feedstocks for biodiesel mean different fatty acid compositions, so these properties will vary for biodiesel produced from different feedstocks. It is then necessary to create a standardization of fuel quality without any difficulties. Austria was the first country to do so, for rapeseed oil methyl esters as a diesel fuel. Guidelines have also been defined in other countries such as Germany, Italy, France and United States. The specifications include the American Stars for Testing Materials (ASTM 6751)[10] or the European Union (EN 14214) [11]Standards. [6]

Biodiesel's properties are characterized by physicochemical properties that include the following: [6,12]

Table 1.2 – Physicochemical properties of biodiesel and their general descriptions

Parameter	Description
Kinematic Viscosity	Thickness of the oil, and the most important property of biodiesel. Influences the operation of fuel injection equipment, especially at low temperatures when an increase in viscosity affects the fluidity of the fuel. High viscosity leads to the formation of soot and engine deposits due to insufficient fuel atomization.
Cloud and Pour Points	Cloud Point - Temperature at which the fuel becomes cloudy due to formation of wax crystals. Pour Point - Temperature at which the amount of wax formed is sufficient to gel the fuel – in other words, the lowest temperature at which the fuel can flow.
Cold Filter Plugging Points	The temperature at which the test filter starts to plug due to fuel components starting to gel or crystallize, representing the fuel's limit of filterability. Another indicator of low temperature operability of fuels. CFPP of a fuel is usually lower than the cloud point.
Flash Point	Temperature at which the fuel will ignite, if exposed to a flame or spark, and the lowest temperature at which fuel emits enough vapors to ignite.
Cetane Number	Directly related to the inflammability of the fuel, therefore, to reaction efficiency within the engine. High CN implies a short ignition delay, while low CN tends to result in incomplete combustion, increasing gaseous and particulate emissions. Based on two compounds: hexadecane (CN of 100) and heptamethylnonane (CN of 15).
Density	Gives an indication of the delay between injection and combustion (ignition quality) and the energy per mass (specific energy). Oils that are denser contain more energy.
Acid Number	Indicator of carboxylic acid groups concentration in a compound. The amount of KOH required to neutralize a certain amount of biodiesel. Neutralization can be necessary since high acid content can cause serious corrosion in the engine.
Carbon Residue	Carbon residue can form by decomposition and pyrolysis of the fuel components, and can clog the fuel injectors.
Calorific Value	Energy released as heat during combustion.
Sulfur content	There are emissions of sulfur oxides if there's sulfur in the fuel.
Sulfur Ash Content	Amount of inorganic contaminants such as abrasive solids and catalyst residues, and also of soluble metal soaps in a fuel sample.
Water and Sediment Content	Biodiesel can take up a greater amount of water than diesel fuel, either dissolved or in suspended water droplets. Water reduces the heat of combustion and causes corrosion of engine components. Fuel oxidation can also create insoluble compounds that might create rust and dirt particles.
Free and Total Glycerin	Measurement of how much of the glyceride content remains unconverted into alkyl esters. Total glycerin is calculated from the amount of free glycerin, monoglycerides, diglycerides and triglycerides.
Phosphorus, Calcium and Magnesium content	Minor components typically associated with phospholipids and gums that may act as emulsifiers or cause sediment, resulting in lower yields in the biodiesel production process.
Moisture Impurities and Unsaponifiables (MIU) Content	Amount of water, filterable solids (bone fragments, food particles or other solids) and other non-triglycerides in an oil which cannot be converted. They must be removed before biodiesel production or during ester purification. Moisture can react with alkaline catalysts if such are used in biodiesel production, forming soap and emulsions.
Lubrication properties	Biodiesel is stated to have improved lubrication characteristics, but those can contribute to the formation of deposits and plugging of filters. It depends mainly on degradability, glycerol (and other impurities) content and cold flow properties.
Oxidation Stability	An indication of potential reactivity with air, and can determine the need for antioxidants. Oxidation occurs due to the presence of unsaturated fatty acid chains that can react with air, leading to a greater susceptibility of biodiesel to oxidative degradation then fossil diesel fuel.
Iodine Index	The grade of oil unsaturation. Biodiesel produced from highly unsaturated fatty acids containing oils (high index) are less viscous show greater cloud point and pour points which make the biodiesel more suitable for cold weather conditions. However, it is also prone to oxidation, has a lower cetane index and lower combustion heats. The CN of such biodiesel is generally higher than diesel fuels. In contrast, with a low index, due to oils with a high proportion in long chain fatty acids (18C) produced biodiesel has a higher CN and combustion heat, but also lower cloud and pour points and greater viscosity.

The fatty acid profile of the oil, that is, the type of fatty acids that exist in the triglycerides used for biodiesel production, influences the quality of the biodiesel produced.

Among the many vegetable oils available, those with a high content in oleic acid are the most suitable due to the greater stability of their alkyl esters and their better characteristics as fuels. [7]

The specifications of diesel and biodiesel fuels are as follows: [11,12]

Table 1.3 – ASTM and EN specifications of diesel and biodiesel fuels

Property	Unit	Diesel (ASTM)	Biodiesel (ASTM)	Biodiesel (EU)
Ester Content	% (m/m)	-	-	96.5
Flash point	ºC	60 to 80	130 min.	101 min
Cloud point	ºC	-15 to 5	-3 to -12	-
Pour point	ºC	-35 to-15	-15 to -16	-
Cold filter plugging point	ºC	-8	5 max	-
Cetane number	-	46	47 min.	51 min.
Density at 15ºC	Kg/m^3	820-860	880	860-900
Kinematic viscosity at 40ºC	mm^2/s	2,0-4-5	1,9-6,0	3,5-5,0
Iodine index	g I_2/100g	-		120
Acid number	mg KOH/g	-	0,5 max	0,5 max
Oxidation stability	Depends on test method	25 mg/L max	-	3h min
Carbon residue	% m/m	0,2 min	0,050 max	0,3 max
Sulphur Ash Content	% m/m	-	0,002 max	0,02 max
Water and sediment	-	0,05 max	0,005% vol max	500 mg/kg max
Methanol	-	-		0,2 max
Monoglycerides	% m/m	-	-	0,8 max
Diglycerides	% m/m	-	-	0,2 max
Triglyceries	% m/m	-	-	0,2 max
Free Glycerin	% m/m	-	0,02 max	0,02 max
Total Glycerin	% m/m	-	0,24	0,25
Phosphorous	% m/m	-	0,001 max	-
Lubricity	m	0,460 mm max	520 max	-

1.3. Importance of feedstock, and food vs. energy

Of all the factors contributing to the cost of biodiesel, feedstock is considered the most important, since its cost can reach around 70% of the total operating cost. Thus, the most suitable feedstocks are those with the greatest amounts of extractable oil. However, costs are reduced due to the non-existing need to refine crude oil completely to produce biodiesel. [7]

In general, biodiesel feedstock can be divided into four main categories, with the following examples: [6]

1. Edible vegetable oil: rapeseed, soybean, peanut, sunflower, palm and coconut oil.
2. Non-edible vegetable oil: jatropha, karanja, sea mango, and halophytes.
3. Waste or recycled oil.
4. Animal fats: tallow, yellow grease, chicken fat and by-products from fish oil.

Globally, there are more than 350 oil-bearing crops identified as potential sources for biodiesel production. The most suitable feedstocks must be either low cost and/or have high oil content.

The feedstock's origin itself is tightly related with its sustainability, since while biodiesel is usually seen as an environmentally friendly fuel, its demand can impact global agriculture, resulting in a "food vs. energy" dilemma, which leads to less public acceptance of biodiesel. A possible solution is the use of waste or non-edible fats and oils. [9]

1.3.1 Non-edible vegetable oils

Non-edible vegetable oils are known as the second generation feedstocks and are considered promising substitutions for traditional edible food crops for the production of biodiesel. As the name implies, they are unsuitable for human consumption, due to the presence of toxic components in the oils. [12]

Non-edible oil plants are usually well adapted to arid or semi-arid conditions and require low fertility and moisture demand to grow. They are commonly propagated through seed or cuttings, so non-edible biodiesel crops could use lands that are largely unproductive – poverty-stricken areas, degraded forests, cultivators' field boundaries, fallow lands, and along roads, railways and irrigation canals. Because of this, the plants have the potential for non-competition with food crops since they can grow in places where food crops wouldn't. Non-edible biodiesel development can as such be a major poverty alleviation factor, since it provides energy security to rural areas and upgrades the rural non-farm sector by restoring degraded lands, and fixing of up to 10ton/ha/year of CO2 emissions. [12]

Conversion of non-edible oil into biodiesel is comparable to conversion of edible oils into biodiesel in terms of production and quality. Some advantages of non-edible feedstocks are

the fact most non-edible plants are highly pest and disease resistant, and their oils have higher heat contents, lower sulfur contents and lower aromatic contents. Also, non-edible feedstocks can produce useful by-products during the conversion process, which can be used in other chemical processes or burned for heat and power generation. For instance, the seed cakes after oil expelling can be used as fertilizers for soil enrichment. [12]

It should be pointed out that global biodiesel feedstocks should not rely on few sources as there could be harmful influence in the long run, like the world's dependence on fossil fuels. The feedstocks should be as diverse as possible, depending on the geographical location. Several potential tree borne oil seeds (TBOs) and non-edible crop source have been identified as suitable feedstock for biodiesel. Some examples are as follows: [12]

- *Nicotiana tabacum* (Tobacco)
- *Jatropha curcas* (Barbados nut)
- *Aleurites moluccana* (Candle nut tree)
- *Pachira glabra* (French peanut)
- *Hevea brasiliensis* (Rubber seed)
- *Sapinus mukorossi* (Soapnut)
- Rice bran

However, there is a wide gap between potential and actual production of biodiesel using non-edible oils of forest origins. There is limited availability of the quality planting material (seed), as well as unreliable marketing channels. The collection of the crops has to be made on scattered locations, with forest plantations presenting picking challenges, and many plants tend to have high dormancy periods. Finally, there is a lack of post-harvest technologies, non-remunerative prices and an absence of state incentives promoting biodiesel damages the cost-benefit ratio. [12]

Additionally, potential of converting non-edible oil into biodiesel requires careful examination. This is because physical and chemical properties of biodiesel produced from any feedstock must comply with the limits of ASTM and EN specifications for biodiesel fuels. In short, production of biodiesel from non-edible oils is a viable pathway to overcome the problems associated with edible oils, but there are several challenges to be tackled. [12]

1.3.2 Waste cooking oil

Waste cooking oil, (WCO), is a post-consumer residue without commercial value, with 29 million tons being generated every year, and being readily available. [9]Thus, biodiesel derived from waste cooking oil could be a viable, plentiful biofuel. [13]

Enweremadu *et al.* (2010)[13] have demonstrated that engine performance using biodiesel produced from WCO is the same as when using biodiesel produced from virgin oils, with no need for engine modifications. Overall, the engine performance of WCO-based biodiesel was only marginally poorer than diesel. NOx emissions were slightly higher while CO emissions, particulate matter emissions and smoke intensity were lower.

1.3.3 Microalgae

Despite their potential, second generation feedstocks may not be plentiful enough to satisfy the global energy demand. Also, biodiesel derived from vegetable oils and animal fats have a relatively poor performance in cold weather. For animal fats, a high amount of saturated fatty acids makes transesterification difficult. And in case of waste cooking oil, collection infrastructure and logistics could be a hurdle as the sources are very scattered. [6]

Microalgae have recently emerged as the third generation feedstock, and have become the latest potential inexhaustible source of biodiesel. Microalgae are prokaryotic or eukaryotic photosynthetic microrganisms – examples include the prokaryotic Cyanobacteria (*Cyaniphyceae*) and the eukayotic green algae (*Chlorophyta*).[14] Microalgae have a high photosynthetic efficiency, growth rate and productivity compared to conventional crops and are easier to cultivate than many other types of plants, while requiring much less land area. [15] Microalgae can have very high lipid contents (up to 70% of the dry biomass) but higher lipid productivity is achieved with many species with lower maximum lipid contents (between 20 to 50% of the dry biomass). The potential biodiesel production rate of microalgae can be greater than 120000 kg of biodiesel/ha/year. [14]

Other advantages of microalgae include potential for wastewater treatment by using water contaminants (such as NH_4^+, NO_3^-, PO_4^{3-}) as nutrients, and the fact the remaining algae biomass can be processed into ethanol and methane, and used as organic fertilizer. [14]

Microalgae are thus very economical compared to edible oils and appear to be a potential source of renewable biodiesel capable of meeting the global demand for transport fuels. The main obstacle remains its high production cost. [15]

1.4. Oil extraction technologies

After acquisition of the oil containing feedstock, the oil must be extracted before being processed into biofuel. There are three main methods for extraction of the oil: mechanical extraction, solvent extraction and enzymatic extraction. [12]

Mechanical extraction is the most conventional practice, involving a machine (manual ram or engine driven screw press) to crush the feedstock and let the oil out. Such methods require further filtration and degumming steps, but the final yield of these methods can go up to 80%. A problem associated with mechanical extraction is that the design of the extractor must be suited to some particular seed, so yield can be affected if other seeds are used. Pre-treatment of seeds, such as cooking, can increase the oil yield up to around 90%. [12]

Solvent extraction involves the use of a liquid solvent. There are three main methods of this type: Hot water extraction, Soxhlet extraction and ultrasonication technique. The liquid chosen should be a good selective solvent and its viscosity should be sufficiently low to circulate freely. Temperature, particle size, and agitation all affect the extraction rate, as the solubility of the material will increase with the increasing temperature, a small particle size is preferable as it results in a greater interfacial area between solid and liquid, and agitation increases the diffusion and therefore the transfer of material from the surface of the particles. Extraction methods using n-hexane method results in the highest oil yield (95-99%) which makes it the most common type employed. Solvent extraction is only economical for a large-scale production (more than 50 tons of biodiesel per day), it is a very time consuming technique compared to other types and has a negative environmental impact as a result of the wastewater generation, higher energy consumption and higher emissions of volatile organic compounds resulting in human health impacts. [12]

Enzymatic extraction has emerged as a promising technique for extraction of oil. It involves the use of enzymes (alkaline proteases, hemicellulases and cellulases) to hydrolyze structural polysaccharides and proteins, thus releasing any lipids that are then more easily recovered by any solvent based extraction steps. It is environment friendly and does not produce volatile organic compounds, but it is very time consuming and as mentioned, it still needs an actual extraction step to isolate the oil. [6]

1.5. Production of biodiesel

Crude vegetable oils can't be directly used in conventional diesel engines, with high viscosity, low volatility and polyunsaturated species being the main barriers. [6]There are several techniques to produce usable fuel from various oil feedstocks, including: Pyrolysis, Microemulsification, Dilution and Transesterification. [12]

Pyrolysis (thermal cracking) is the conversion of organic matter in the absence of oxygen and in presence of a catalyst and heat. Thermal decomposition of triglycerides produces alkanes, alkenes, aromatics and carboxylic acids, with the liquid fractions having some similarity with diesel fuel. The product has lower viscosity, flash point and pour point than diesel fuel and equivalent calorific values, but lower cetane number – resulting in more smoke emissions than diesel. The pyrolyzed vegetable oils usually contain acceptable amounts of sulfur, water content, copper corrosion values and sediments but unacceptable amounts of ash and carbon residue. [12]

Microemulsification is another reliable approach to solve the problem of high viscosity of vegetable oils. A microemulsion is a transparent, in equilibrium, thermodynamically stable colloidal dispersion of microstructure with diameter ranges from 100 to 1000Å. Microemulsion can be made of vegetable oils with an ester and dispersant (co solvent), or of vegetable oils, and alcohol such as ethanol, ethanol, butanol, hexanol and a surfactant and a cetane improver, with or without mixture with diesel fuels. [12]

Also, the oil can simply be diluted with diesel to reduce viscosity and improve performance of the engine. No chemical process is involved, and the method generally produces good results. [12]

Finally, synthesis of fatty acid alkyl esters (biodiesel) is the more conventional method, known as transesterification. [12]

1.5.1 Transesterification

Transesterification, or alcoholysis, is the reaction of alcohol with the oil. [12] The triglycerides (or triacylglycerols) are converted into alkyl esters, known as Fatty Acid Methyl Esters (FAME) if the alcohol used is methanol or Fatty Acid Ethyl Esters (FAEE) when the alcohol is ethanol. The resulting esters have lower viscosity than the reagent oil, thus are more suited for diesel engines. Triglycerides, being esters of fatty acids with glycerol, are converted into fatty acids esters of smaller alcohols, thus releasing glycerol, since short-chain primary alcohols are used, that are unable to bond with more than one fatty acid. Glycerol is an important by-product and can be burned for heat or used as feedstock in, for instance, the food

and cosmetic industry. [6] The transesterification of oil triglycerides is the most common technology of biodiesel production, and is seen to have advantages over other approaches, due to high conversion efficiency and low cost. [9]

The reaction is summed up in the following figure:

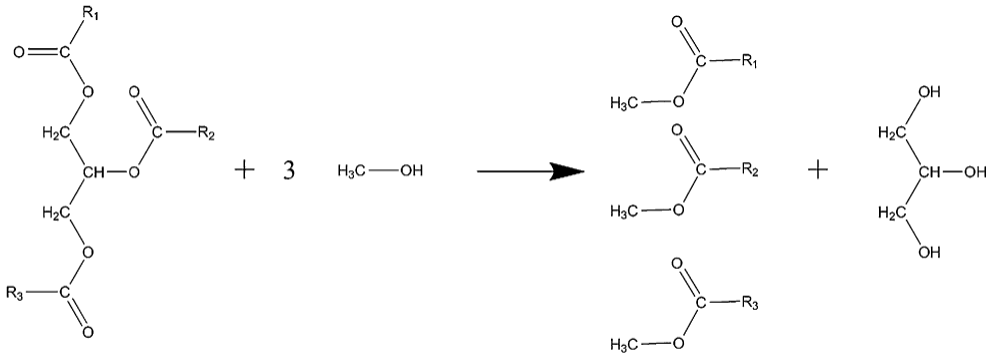


Figure 1.1 – Example of a transesterification reaction between a triacylglycerol and 3 methanol molecules, producing 3 fatty acid methyl ester (FAME) molecules and glycerol. Source: Rodrigues *et al* (2001)[16]

Transesterification consists of a number of consecutive, reversible reactions, with the triglycerides being converted step wise to diglycerides, monoglycerides and finally glycerol. In each of the three reactions, the R group of the alcohol (CH_3, in methanol's case) is replaced by the glycerol backbone and any fatty acid groups still attached to it. Triglycerides, being esters of fatty acids with glycerol, are thus converted into fatty acids esters of smaller alcohols, with glycerol being released. In other words, one of the fatty acid groups is bonded with the alkyl group of the alcohol, with the glyceride losing said fatty acid group, and getting an OH group in its place. Because of the bonding of the OH group and the acylglycerol (glyceride), the alcohol is referred to as an "acyl acceptor". Since short-chain primary alcohols are used, and these are unable to bond with more than one fatty acid (glycerol is a tertiary alcohol, and can esterify with 3 fatty acids), in each reaction only one product ester is produced at the price of one alcohol molecule. The reaction goes on until there are no more fatty acid groups left in the glyceride, leaving a free glycerol. The stoichiometric ratio for the complete reaction thus consists of 1:3:3:1 for the Triacylglyceride, Alcohol, Fatty Acid Ester and Glycerol, respectively.[6]

$$\text{Triglyceride} + \text{Methanol} \rightarrow \text{Diglyceride} + \text{Fatty Acid Methyl Ester}$$

$$\text{Diglyceride} + \text{Methanol} \rightarrow \text{Monoglyceride} + \text{Fatty Acid Methyl Ester}$$

$$\text{Monoglyceride} + \text{Methanol} \rightarrow \text{Glycerol} + \text{Fatty Acid Methyl Ester}$$

$$\text{Global Reaction: Triglyceride} + 3\ \text{Methanol} \rightarrow 3\ \text{Fatty Acid Methyl Esters} + \text{Glycerol}$$

Figure 1.2 – The three steps of transesterification reaction, with Methanol and FAME as examples

Transesterification reaction is affected by various parameters depending upon reaction conditions. If the parameters are not optimized, yield and reaction completion is reduced. The most important are:

- Free fatty acids, moisture and water content
- Type of alcohol and molar ratio employed
- Type and concentration of catalysts
- Reaction temperature and time
- Rate and mode of stirring
- Purification of the final product
- Mixing intensity
- Effect of using organic co-solvents
- Specific gravity

Transesterification can be performed with or without a catalyst, with catalytic transesterification being the preferred route to biodiesel. [9]

Non-catalytic transesterification methods include the use of supercritical alcohols, which enhances alcohol solubility in the triglyceride phase, and allows for a complete reaction in a very short time, and easy purification of biodiesel and glycerol recovery, but there are high costs involved due to the reactor's costs and high methanol consumption. [6]

1.5.2 Catalytic transesterification methods

In general, catalytic transesterification has some problems such as high time consumption, reaction time lag caused by alcohol's low solubility in the triglyceride phase and the need for separation of the catalyst and impurities from biodiesel. The more conventional catalytic methods involve the use of alkaline or acid catalysts.

The alkaline catalytic method is the fastest and most economical method, being faster than the acid method and achieving high purity and yield of biodiesel, and thus dominating the current production methods. Alkaline catalysts include NaOH, $NaOCH_3$, KOH, $KOCH_3$ and

K_2CO_3, with sodium or potassium hydroxide being the most used, both performing equally well. While sodium and potassium methoxides return better yields, they are very costly, thus not as cost-effective. [6] If the oil feedstock has a high water content, hydrolysis of the triacylglycerols can occur, forming free fatty acids (FFA), which is undesirable. The reaction can only take place if the FFA level is below a desired limit (ranging from 0,5-3%). If not, the free fatty acids are prone to react with the catalyst, forming soap, consuming the catalyst and lowering final ester yields.[9] Other drawbacks include this method being energy intensive (due to the need of high temperatures, with 333-353 K being recommended for a better yield), a difficult recovery of glycerol, and the need to remove the catalyst from the product with the required treatment of alkaline wastewater. [6]

Acid catalysts are more tolerant of free fatty acids, so acid catalysis is the recommended process when the starting materials are low grade or have a high concentration of free fatty acids, and they can be used in conjunction with alkaline catalysts in a two stage process allowing the use of low-cost feedstock like waste oil with high content of free fatty acids. [7] Acid catalysts include sulfuric, hydrochloric, ferric sulfate, phosphoric and organic sulfonic acids. In general, the acid catalyzed reaction is slower but gives very high yields and is less energy consuming, so it also a viable catalytic method. [6]

1.5.3 Enzymes as alternative catalysts - Lipases

The environmental impact of using chemical catalysts, including the large amounts of water needed to wash the fuel and neutralize the pH, has drawn attention towards the use of enzymes. They work under milder reaction conditions, have lower energy requirements, needing temperatures of 293-323 K. Enzymes are also more selective, while having a wide specificity of reagents – it can both transesterify triglycerides and esterify FFA – and produce highly pure glycerol, with a higher commercial value, thus lowering biodiesel production costs. Finally, enzymes do not form soaps. [9]

Lipases (EC 3.1.1.3) are the type of enzymes suitable for biodiesel production. They are hydrolases that can catalyze the transesterification of carboxylic esters, and have excellent catalytic activity and stability in non-aqueous media. [17] However, the reaction rate with lipases is slower than with alkaline catalysts, and there are risks of enzyme inactivation due to methanol (later explained) and due to glycerol accumulation covering the lipase. But while lipases have the potential to be reused and applied at industrial scales, they are currently very expensive compared to alkaline catalysts. [7]

Another important property of lipases is that in a homogenous medium, they have their active centre secluded from the medium by a polypeptide chain called “lid”. In the presence of any hydrophobic interface (oil, a hydrophobic support, or even other lipases), the lid moves to

permit the interaction between its hydrophobic face and the hydrophobic residues that usually surround the lipase active centre with this hydrophobic surface. This way, the lipase becomes adsorbed on this hydrophobic surface (open form), and the active centre is exposed to the reaction medium. This mechanism of action is usually called "interfacial activation". The flexibility of the active centre makes lipases very sensitive to any changes in the experimental conditions, such as the immobilization strategy employed, making it possible to greatly alter their properties. [18]

The catalytic mechanisms of lipases are mostly similar to those of serine proteases. A catalytic triad of Ser-His-Asp/Glu is involved in the cleavage of ester bonds. [19]

1.5.3.1 Lipase Possibilities

When selecting a lipase, the most desired characteristics one has to look for are: [20]

- Their ability to transesterify all mono, di, and triglycerides as well as to esterify free fatty acids
- Low product inhibition;
- High activity and yield in non-aqueous media;
- Low reaction time;
- Reusability of immobilized enzyme;
- Temperature and alcohol resistance.

Several lipases that allow for biodiesel yields of over 90% with a variety of oils and acylation agents have been identified, such as lipases from: [9]

- *Pseudomonas fluorescens*
- *Candida rugosa*
- *Rhizomucor miehei*
- *Thermomyces lanuginosus*
- *Candida antarctica*

Mixtures of enzymes can also be used.

1.5.3.2 Immobilization of lipase

The generally high costs of enzymes are barriers for industrial applications. In order to decrease the costs, the enzyme can be immobilized in a suitable carrier and reused. Immobilized enzymes are defined as "enzymes physically confined or localized in a certain defined region of

space with retention of their catalytic activities, and which can be used repeatedly and continuously". [17]

Support materials play an important role in the usefulness of an immobilized enzyme. The immobilization process should be low-cost and provide adequate large surface area together with the least diffusion limitation in the transport of substrate and product for enzymatic reactions. [21] There are several methods for lipase immobilization, including adsorption, covalent bonding, entrapment, encapsulation, and cross-linking. [17]

Adsorption is the attachment of lipase on the surface of the carrier by weak forces such as hydrophobic interactions and van der Walls forces. It can be prepared under mild conditions without major activity loss and the associated process is relatively easy and low cost. The carrier is also easily recoverable for repeated immobilization. With all these advantages, adsorption is still the most widely employed method for lipase immobilization. However, because of the weak adhesion forces involved, there is the risk of the lipase being stripped off from the carrier. The carriers used include acrylic resin, textile membrane, polypropylene, celite and diatomaceous earth. In general, this technique allows for biodiesel yields higher than 80% with vegetable oil or waste cooking oil as feedstock. An example of a lipase used for large scale industrialization immobilized using this method is lipase from *Candida antartica*, immobilized on acrylic resin, known commercially as Novozym 435, by Novozymes, a Danish company. It can reach yields of over 90% while being stable for as much as 500 hours with various substrates and solvents. [17]

Covalent bonding of lipase to a solid carrier has the expected advantage of irreversible binding of the lipase to the support matrix due to the strong forces involved. But the preparation conditions are rigorous, and the lipase might lose some activity during the process. [17]

Entrapment involves the capture of the lipase within a matrix of polymer. The enzyme is not attached to the polymer, merely has its free diffusion restrained. It is a fast, cheap and easy process, usually involving mild conditions. But the resultant yields from the enzymes are relatively low, due to the mass transfer restriction, so the lipase is only effective for low molecular weight substrates. [17]

Cross-linking the lipase molecules with a glutaraldehyde carrier results in a strong interaction and stability between the lipase and the carrier, but the conditions are intense and the mechanical strength of the immobilized lipase is low. In other words, just like covalent bonding, it can result in some loss of lipase activity. [17]

1.5.3.3 Acyl acceptors, and lipase inactivation caused by alcohols

Methanol and ethanol are generally similar in terms of viability as acyl acceptors for biodiesel production. Methanol is often the preferred alcohol due to lower price. However,

ethanol can be produced at large scale from biomass waste at competitive prices, so it is also a viable acyl acceptor for large scale production. [9]

For biodiesel synthesis, at least a stoichiometric amount of alcohol is required for the complete conversion of triacylglycerols to their corresponding fatty acid esters (a 3:1 ratio) [17] but a molar excess of alcohol over triacylglycerols is required to reach high yields of transesterification – by shifting the reaction equilibrium towards biodiesel production – while at the same time, excess methanol does not favor enzyme integrity. [16] The same happens for ethanol, although in a lesser extent. [7] The inactivation of the enzyme by methanol or ethanol is major obstacle for enzymatic biodiesel production, and to solve this problem, there are three options: methanol stepwise addition, acyl acceptor alterations and solvent engineering. [17]

The stepwise addition of methanol involves adding methanol into the reaction system in several small doses over time, instead of a large amount added at once. Because it is the insoluble methanol that inactivates the lipase, having lesser amounts of methanol in the system at each time while having a total amount of methanol equal to at least the stoichiometric amount for the complete reaction, this is a relatively easy and simple method that allows for high yields. A continuous pumping of methanol into the reactor also allows for similar results, for the same reasons. [17]

Methanol can be replaced by a different acyl acceptor such as methyl acetate or ethyl acetate. Methyl acetate allows for high yields, but has a high cost, and is slower to react, creating major constraints for industrial applications. [17]

Finally, improving methanol solubility via solvent engineering is another reasonable angle to exploit. T-butanol is a good solvent of methanol, and there are several studies involving it with good results. Other solvents such as ionic liquids were also examined, but they are disadvantageous because of high cost, environmental concerns, and the requirement of the separation of the solvent from the reaction medium. [17]

1.5.3.4 Water content

Water plays multiple roles in lipase-catalyzed biodiesel production with non-aqueous media. It has strong influence on the catalytic activity and stability of the lipase. Water is needed to keep the enzyme active in organic solvents, but it might take part in the transesterification, thus influencing the equilibrium. [17]

The unique feature of lipases is that they act at the interface between aqueous and organic phases, so the lipase activity generally depends on the interfacial area. Water facilitates an increase in the available interfacial area, thus it helps to maintain lipase activity. But, excess water might make the lipase more flexible and lead to some unintended side-reactions such as hydrolysis. [17]

Consequently the optimum water content required to maximize enzymatic activity can be determined for most lipases, and the amount for a certain reaction depends on the feedstock, the lipase, the immobilized support and the organic solvent employed. [17]

1.6. Biodiesel purification

After synthesis, biodiesel products need purification to meet the corresponding product standards. The impurities crude biodiesel can contain include free fatty acids, water, alcohol, glycerides, glycerol and metals (and soap if alkali catalysts were used). [22]

Glycerol and biodiesel are not miscible, and because glycerol has a higher density (1050 kg/m^3) than biodiesel (around 880 kg/m^3), simple techniques such as gravitational settling or centrifugation allow for a good phase separation. If a solvent is used, phase separation can occur after solvent recovery, which can be done by conventional evaporation or distillation. [22]

Conventionally, biodiesel is purified by washing with distilled water. A large amount of water is usually consumed in order to remove soap and other contaminants, and also to reduce the alkaline metal (Na, K) concentrations. Purification of biodiesel produced with immobilized lipase is less complex then with biodiesel produced with alkali or acid catalysts because the absence of such metals and mineral acids make it easier to purify the biodiesel. Free fatty acids, while causing significant emulsion of the system, can be removed during the reaction, since lipases can use them as substrates. [22]

Another purification technique is the use of solid adsorbent or ion-exchange resins, a process referred to as “dry wash”. Membranes have also been used for biodiesel production. These exhibit several advantages over the conventional ones, such as minimization of capital cost and high specific area of mass transfer, and membrane separation can be conducted simultaneously with the transesterification using membrane reactors. However, while these processes look promising on industrial scale, they still need optimization while considering the water, energy and operation costs. [22]

1.7. Bioreactors

Most of the immobilized lipase (IL) catalyzed biodiesel productions in lab are performed in shaking flasks or very small reactors, but for a larger-scale operation, the reactor must be carefully designed. Several types have been used for biodiesel production, such as stirred tank reactor (STR) and packed-bed reactor (PBR). [22]

A stirred tank reactor is a well-mixed reactor. It is the most often used for bioprocesses at different scales because of the ease of construction, operation and maintenance. STRs can be operated in both batch and continuous modes, with Batch STRs being usually used in small scale. Good mixing can improve contact between substrate and biocatalyst and provide a good

dispersion, reducing mass transfer resistance and overall reaction rate. However, Continuous STRs seem to be more applicable on large scale because of higher productivity. But, one of the major disadvantages of STR is the potential damage to the catalyst due to the shearing force used, thus limiting reusability of catalyst, and thus stirring speed must be optimized. [22]

A packed-bed reactor is usually used for a continuous operation. It is the most promising reactor for industrial-scale production of biodiesel. PBR is basically composed of a column in which ILs are packed – and auxiliary equipment such as a water bath for maintaining required reaction temperature and pumps for transferring reactants. Compared with STR, PBR can obtain a larger reacting surface area per unit volume with associated higher volumetric productivity in continuous industrial processes. The parameters that should be considered for optimizing biodiesel production include flow rate, reaction temperature and alcohol:oil molar ratios. Flow rate is the most important operational variable, with a low flow rate usually desired to obtain a long enough residence time for a higher yield. But long operations (over 7 days) can result in decreased yields due to glycerol accumulation on the ILs. A high pressure drop associated with small carrier size (that can be over 0,1 MPa/m reactor) is also another drawback of PBRs. [22]

Overall, continuous reactors are more advantageous over batch reactors because of the ease of operation, increased enzyme stability, facilitated enzyme reuse, and higher enzyme to substrate ratio, which decreases reaction time, and subsequent separation and cost effectiveness. High cost of the lipase has been the main obstacle to industrial scale commercialization of the enzymatic process but enzyme cost has become more favorable in recent years and optimization of reaction conditions increases the operational stability of lipase, reducing the impact of its high cost by allowing reutilization. [22]

1.8. The need for new technologies: Supercritical Fluids

The majority of the enzymatic reactions for biodiesel synthesis are carried out in batch systems using methanol as the acyl acceptor, in the presence of various solvents such as hexane, t-butanol and also in solvent-free media, employing various lipases. However, in solvent-free systems, mass transfer properties of the substrates are poor, methanol and ethanol have poor solubility in oil, and as described earlier, lipase may be inactivated by excess alcohol. Organic solvent systems are also disadvantageous because of the high cost, environmental concerns and separation issues. Therefore, the need for better yielding, better separation methods for higher purity product and environmentally friendly processes have led to the search for new technologies. [23]

Use of supercritical fluids has been found to be a promising alternative to organic solvents for enzymatic reactions. A supercritical fluid (SCF) is any compound at a temperature and pressure above the critical values (critical points, Tc and Pc, respectively). [24] The fluid is

neither a gas nor a liquid and is best described as intermediate to the two extremes. This fluid retains solvent power approximating liquids as well as the transport properties common to gases, thus differing from ordinary solvents. [24]

Near the critical point, small changes in temperature or pressure lead to significant changes in solubility, partition coefficient, dipole moment and the dielectric constant. The change in properties from subcritical fluid to supercritical state is especially noteworthy for water and carbon dioxide. The two fluids acquire very high solubility and diffusion power, while also allowing high miscibility of gases and also have variable density. [24]

Not surprisingly, SCFs as non-aqueous solvents for enzyme-catalysed reactions have gained the attention of enzymologists since the 1980s and have been employed in a variety of biotechnological applications due to their numerous advantages. Enzymes are not only able to function in SCFs but they also display interesting properties such as: [24]

- Altered substrate specificity and enantiomer selectivity;
- Suppression of side-reactions;
- Increased stability;
- Molecular memory – retention of a favourable transition state by the enzyme after a first reaction, enabling faster following reactions than when the enzyme was in its "default" state.

1.8.1 Supercritical carbon dioxide as a solvent

Carbon dioxide (CO_2) is the most commonly used supercritical fluid ($scCO_2$) due to its advantages:

- Non-flammability, non-reactivity and low toxicity;
- High availability, being processable in more available and less costly equipment;
- Leaves no residues and its recycling mitigates GHG emissions. [16]

Supercritical carbon dioxide can thus serve as solvent for "difficult" chemical transformations, such as the direct reaction of hydrogen and oxygen to form hydrogen peroxide or various selective free-radical reactions.

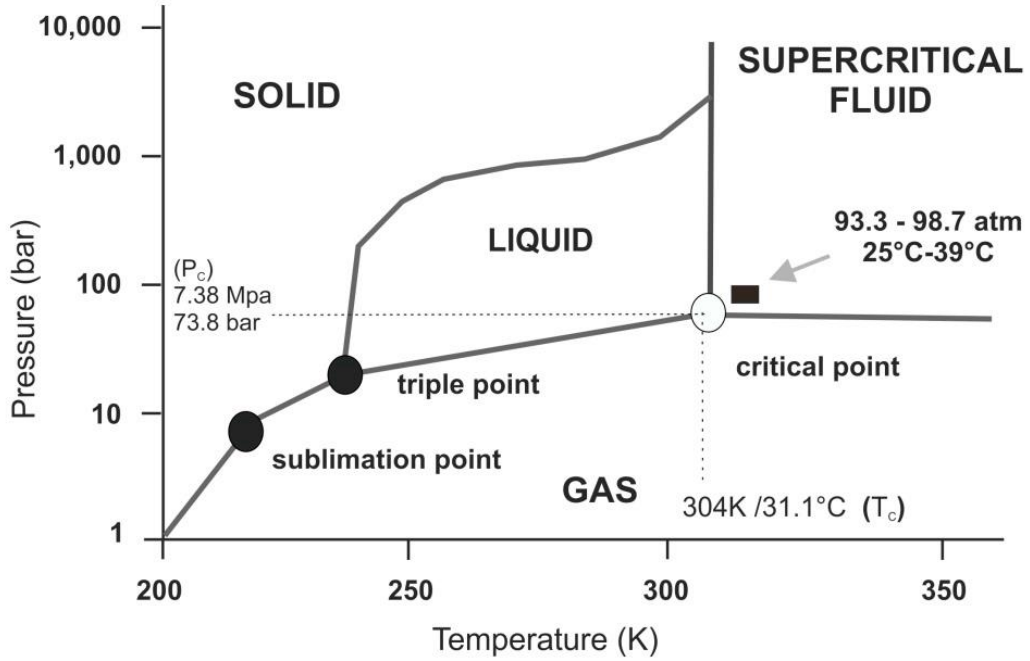


Figure 1.3 – Schematic p-T phase diagram of CO_2, a gas commonly used in supercritical phase. Source: Budisa *et al* (2014) [24]

Another important advantage over other solvents is its ability to diffuse through solid matrices, such as immobilized enzyme preparations, as well as the improved diffusivities of the dissolved components in the reaction media. The solvation ability of $scCO_2$ can be easily modified by adjusting temperature and pressure conditions. Through the manipulation of temperature and pressure, the solubility of a given substance in $scCO_2$ can increase, or decrease to the point of precipitation.[16]

$ScCO_2$ can be applied to biodiesel production in two ways. First, it can be used to extract oil from a feedstock material – like hexane, it is non-polar thus capable of lipid extraction from various sources, with potential for large-scale processes. It should be noted that even in optimized conditions, the oil yield of $scCO_2$ extractions does not quite reach that of hexane. [25,26,27]

$ScCO_2$ also has potential as a solvent for the biodiesel production itself. Having a moderate critical pressure (7,29 MPa) and low critical temperature (304,25 K), $scCO_2$ allows for better extraction of thermally sensitive lipid fractions without degradation. It can selectively extract/recover the alkyl esters from the reaction mixture since the solubility of fatty acid esters in $scCO_2$ is several orders of magnitude higher than the solubility of triacylglycerols and glycerol. [16] This creates the interesting possibility of an integrated oil extraction, biodiesel production and biodiesel purification process, since the same solvent ($scCO_2$) is suitable for all three stages.

1.9. Lipase-catalysed biodiesel synthesis in $scCO_2$

Considering the cheaper alternative of alkaline catalysts, the economic impact of biocatalysis is apparently a disadvantage, but a $ScCO_2$-based integrated extraction/reaction/separation process could change everything. There are several studies reporting lipase-catalysed biodiesel synthesis in $scCO_2$ but many use very small reactors (of a few millilitres) for lab scale experiments, but studies with larger volumes exist. It should be noted that these studies include experimentation with several pressure and temperature conditions, and also residence times, in an attempt to optimize the process using the apparatus and reagents at hand.

Rathore *et al.* (2007) obtained 60-70% conversion ratios using Novozym 435 (*Candida antartica* lipase) for several edible oils – palm oil and groundnut oil - and non-edible oils – *Pongamina pinnata* and *Jatropha curcas*, in a 7 mL batch reactor at 6,8 MPa and 298 K. [28]

Also using a 7 mL batch reactor and Novozym 435, Varma *et al.* (2010) achieved a maximum of 70% conversion, with 10 MPa and 298 K as operating conditions. The feedstocks used were sesame oil and mustard oil. [29]

Lee *et al.* (2009) conducted a study using soybean, olive, palm, rapeseed and sunflower oils and Novozym 435. In a 100 mL batch reactor, the highest yield achieved was 65,18%, but an optimized stepwise reaction system enabled a 98,92% conversion at 6 hours. It should be noted that the immobilized lipase was reusable several times, suggesting applicability of this process for industrial production rates. [30]

Using a continuous system in a 5mL reactor, Jackson *et al.* (1996) achieved a FAME yield of over 98% at 17,2 MPa and 323 K, from corn oil, and with Novozym 435 as a catalyst. This was followed by a complete fractionation of the reaction mixture. The CO_2 flow rate was 1mL/min and the oil flow rate was 4µL/min. [31]

Ciftci *et al.* (2011) achieved a yield of 93,3% in a 23,45 mL continuous reactor at 336,05 K and 19,4 MPa using Novozym 435, and corn oil as a feedstock. [23]

Rodrigues *et al* (2010) implemented a continuous process in which several combinations of enzymes and oils were used. This particular setup used a static mixer (69,37 mL) to mix the $scCO_2$, the oil and the methanol before delivering the mix the packed bed enzymatic reactor (4,3 mL) With virgin sunflower oil, Lipozyme TL IM (*Thermomyces lanuginosus* immobilized on an acrylic resin, sold by Novozymes) a FAME yield of 98,6% was achieved at 20 MPa, 313 K, for a residence time of 20s and an oil to methanol molar ratio of 1:24. The purity of FAME obtained was 93% when the separator was at 333 K and 12 MPa. The same enzyme was less efficient with waste cooking sunflower oil, reaching a yield of 89%, but using a mixture of Lipozyme TL IM and Novozym 435, a yield of 99% was achieved. [16]

Finally, there is a recent study by Gameiro *et al* (2015), where production of FAME was carried out using chicken feather meal, at 313 K and 25 MPa, using Lipozyme RM IM ® as a biocatalyst. This study is particular noteworthy because it was carried out in a pilot plant unit, and the oil extraction and biodiesel production were integrated in a continuous process. The extraction took place in a 7720 mL vessel, with methanol being introduced and mixed with the outlet stream of the extraction vessel. The resulting mix was pumped into a 1178 mL packed-bed enzymatic reactor, where transesterification took place. Various $scCO_2$ flow rates and oil:methanol molar ratios (up to 1:24) were tested, resulting in FAME yields between 96,7% and 98.8%. [32]

Overall, these studies with $scCO_2$ are in accordance with the general tendency regarding biodiesel production in bioreactors: continuous reactors allow for higher yields than batch reactors, as explained earlier.

1.9.1 Techno-economic evaluation of IL-catalysed production of biodiesel using $scCO_2$

Techno-economic evaluation is important to estimating production cost and determining the costliest units for further optimization. The main factors to consider include raw material costs (oil feedstock, alcohol, enzyme and CO_2), process parameters (oil-to-biodiesel conversion ratio, residence time, biodiesel recovery yield, lipase lifetime and CO_2 loss), process design regarding water recycling and heat integration, and by-product (glycerol) credit. [22]

Lipase cost contributes a great part of the total production cost due to their high price, indicating that a very high productivity is required for the process to be cost effective, and that reusability of ILs is important to reduce production cost. A promising solution is to increase the specific activity of IL to decrease enzyme loading, using protein engineering approaches. And future enzyme prices can be lower, as production methods are optimized and scaled-up, thus lowering the impact of lipase on production costs.[22]

Acyl acceptor type and concentration, water content, enzyme loading, alcohol to oil ratio, temperature and reaction media, all affect biodiesel yield and stability of ILs. Optimal conditions greatly depend on oil feedstock and IL employed. The use of CO_2 is a promising technique with very minimal loses, but it incurs substantial expense due to energy requirements (cooling, heating and pumping). [22]

Regarding the combination of lipase and supercritical carbon dioxide technology for industrial scale biodiesel production, there is one economic study that stands out. Lisboa *et al* (2013) recently analysed the economy of a scCO2 based enzymatic process, for the production of biodiesel from waste cooking sunflower oil. In a pilot plant unit, they studied the conversion of WCO to biodiesel and biodiesel recovery. The enzyme used was *Thermomyces lanuginosus* lipase (Lipozyme TL IM), and the acyl acceptor was ethanol. The high pressure apparatus

included a packed bed reactor and two separators connected in series (working at different pressure and temperature conditions), with CO_2 recycling. The biodiesel produced in this study conformed to the EN14214 norm. The data generated was used to envisage an industrial plant, with estimation costs being made considering a conversion of 8000 tons of WCO per year, with a yield of 86,7%. A yield of 99,9% was also considered, but this was found to increase investment costs greatly because of the much bigger reactor required ($3 \times 8{,}6m^3$). The 86,7% yield scenario, requiring the smallest reactors out of the scenarios considered ($3 \times 3{,}4m^3$), was found to lead to the lowest investment cost (14,7 million €). This scenario leads to biodiesel costs of 1,64€/L and 0,75€/L, for a WCO price of 0.25€/kg, ethanol price of 0.84€/L, CO_2 price of 0,20€/kg, and enzymes prices of 800€/kg and 8€/kg, respectively. This last enzyme price corresponds to a optimistically possible future price for enzyme should large scale enzyme production methods be optimized and implemented. The cost of raw materials is mitigated by selling glycerol, glycerol amounts being equivalent to 10% of the total biodiesel, and selling at 0,78€/kg. Without glycerol selling, even the best case scenario resulted in a 0,83€/L biodiesel cost. In short, a combination of biocatalysis and supercritical fluid technology with a cheap feedstock can be competitive compared to current conventional biodiesel production methods, should prices for enzyme and feedstock (main factors in production costs) reach favourable values. [9]

1.10. Olive Oil and its Extraction

Olive tree (*Olea europea*) belongs to the Oleaceae family, and it is the only tree from that family with an edible fruit (the olive). [33] The olive fruit consists of pulp (70-90% of total weight), stone (9-27%) and seed (2-3%), with the main constituents (water and oil) mainly concentrated in the pulp and seed. [34]

Olive oil extraction is one of the most traditional agricultural activities in the Mediterranean region, dating back thousands of years, due to the presence of the oil in Mediterranean diet and its health benefits. [35] The world's production of olive oil is 2.951.800 tons per year, with Spain being the greatest producer in the world – 1.305.400 tons per year (both values correspond to the average from the 2008/2009 to 2013/2014 seasons). [36]

Shown in the following figure is a schematic of olive oil extraction methods.

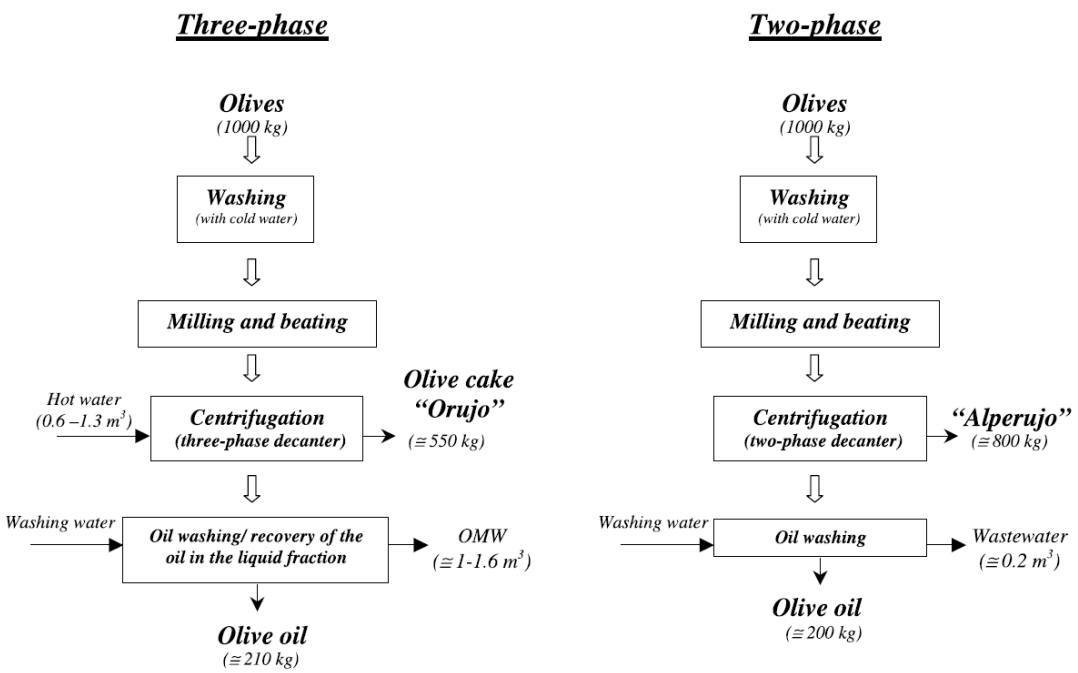


Figure 1.4 – Schematic of the three and two phases methods used in olive oil extraction. Source: Alburquerque *et al* (2004) [34]

Until around 1960, the technology dedicated to that process consisted of a discontinuous press and decantation system, but the technology progressed significantly, and a three-phase centrifugation system appeared. By means of a continuous process, the oil, vegetation water and solid phase (olive cake, or "orujo" in Spain) could be separated. [34] The main inconvenience of this system is the generation of large amounts of olive-mill wastewater (OMW), also known in Spain as "alpechín". It results from the water spent to dilute the olive fruit paste before being introduced in the centrifuge, plus the already existing water in the fruit. [34] "Alpechín" is a stable emulsion of pulp remains, olive oil, mucilage and pectins and its elimination has always been a serious environmental problem due to high phenolic and fatty acid content and to its synergic effect with other organic and inorganic compounds. But the residue has a high quantity of organic matter and macronutrients, thus creating the possibility of using it as a fertilizer, after an efficient way of eliminating its high phenolic and fatty acid loads is applied. Examples include steam-based treatments and separation methods via osmosis. [33]

This method has phased out since the 90's and now most producers use a new two-phase system. Although it is called the "ecological system" because it involves less water and energy consumption, it still produces a solid and humid by-product known as two-phase pomace and also as "alperujo" or "alpeorujo". 800kg of this residue are produced for each 1000kg of olives. Just in Spain, based on its olive oil production, that means that over 5.000.000 tons of alperujo are produced every year. This residue presents new challenges to the olive industry

because it is also environmentally problematic but cannot be treated in exactly the same way as the residue from the three-phase system. [33]

1.10.1 Alperujo

Olive Pomace, or Alperujo, has a variable composition, depending on the olive variety and the processing methods. It is a dark mixture, with an intense smell, a moderately acid pH and a high conductivity. [35]

Water composes most of the residue (65%) and around half of its organic composition is lignin, which difficults the degradation of alperujo by microorganisms and their enzymes. Mannitol, sucrose and fructose are present, forming at the same time a good base for microorganism growth. There are also cell wall remains (from the olive) with a considerable amount of pectin polysaccharides and hemicellulose polymers rich in xylene and xyloglucans. Polyphenolic compounds, oils and proteins account for a small fraction of its total organic composition. [33]

Table 1.4 – Main components of the organic fraction of the alperujo residue, as per the studies of Albuquerque *et al*. (2004)[34], involving several alperujo samples from different plantations

Material	Percentage
Lignin	32-55%
Hemicellulose	27-41%
Cellulose	14-25%
Fats	7-19%
Protein	4-11%
Water-soluble carbohydrates	1-16%
Water-soluble phenols	0,6-2%

Alperujo cannot be directly scattered on the soil, since that leads to serious ecological problems. It has a phytotoxic activity that does not allow plants to germinate. Alperujo contains the same contaminant polyphenolic load as olive-mill wastewater, and also a significant lipid fraction that together, account for the antimicrobial effects currently attributed to olive-mill wastes and by-products. In fact, the polyphenolic compounds in alperujo could have pharmacological applications, as explained further on. [34]

1.10.1.1 Phenolic constitution of Alperujo

Phenols have one or several aromatic rings (monomeric phenols or polyphenols, respectively) with hydroxyl groups or with functional derivates like ester, methyl ester and glycoside. These types of compounds can be found in plant tissues, as primary metabolites in protein, nucleic acid, or carbohydrates synthesis. They can also be secondary metabolites, many with an unclear function. Phenols, and polyphenols in particular, can be oxidized very easily, thus serving as good antioxidants. Phenols can serve as activators or inhibitors of plant growth, seed germination, photosynthesis, nutrient absorption, and dry matter accumulation in plant roots and vestiges – hence their phytotoxic properties. Phenols can also accumulate in the surfaces of plant tissues, and act as UV light blockers, protecting the plant from UV light's harmful effects. Finally, they can also have an antimicrobial property, protecting the plant from microorganisms. [33]

Olives have a large number of phenolic compounds. These have an enormous antioxidant potential and contribute to the nutritional value of olives and olive oil. An antioxidant rich diet can provide protection from oxidant stress related diseases, like cancer and arteriosclerosis. They also prevent lipid oxidation, thus slowing down food deterioration. [33]

Olive phenolic composition varies with several factors: olive variety, maturity state and climate conditions. In the olive, the following compounds can be found:

Table 1.5 – Possible phenolic compounds that can be found in olives. Source: Alvarado *et al.* (2008) [33]

Type	Name
Phenylethanoids	Oleuropein
	Tyrosol
	Hydroxytyrosol
Cinnamic acid derivates	Ferulic acid
	Caffeic acid
	p-Coumaric acid
Benzoic acid derivates	3,4-dihydroxyphenylacetic acid (DOPAC)
	4-hydroxybenzoic acid (PHBA)
Benzenediols	Catechol (Benzene-1,2-diol)
	Methyl catechol
Flavonoids	Cynaroside (Luteolin-7-O-glucoside)
	Apigetrin (Apigenin-7-O-Glucoside)
	Quercetin
	Rutin (Quercetin-3-O-rutinoside)
Anthocyanins	Chrysanthemin (Cyanidin-3-glucoside)
	Antirrhinin (Cyanidin-3-rutinoside)

Hydroxytyrosol, or 3,4-dihydroxyphenyl ethanol, and also known as 4-(2-Hydroxyethyl)-1,2-benzenediol (IUPAC name) has a chemical formula of $C_8H_{10}O_3$, and a molar mass of approximately 154 g/mol. It is formed from the hydrolysis of oleuropein, a bitter glycoside usually removed from olives. Oleuropein is a glycosylated tyrosol ester of elenolic acid. It corresponds to the union of elenolic acid, hydroxytyrosol and glucose. Oleuropein can constitute at least 14% of the olive's dry weight, but during the olive's maturation, much of it hydrolyses. Thus, hydroxytyrosol represents the main phenolic compound in the olive, whether in free form or conjugated, with some varieties of olive having up to 760 mg/kg. [33]

Due to their polarity, most phenols remain in the aqueous phases during olive oil extraction, but not all, given how they are also partly amphipathic. Residues such as OMW ("alpechín") and Alperujo can then have these substances in large amounts. Another important source of hydroxytyrosol and oleuropein is the olive leaf, currently commercialized as a nutrient supplement. [33]

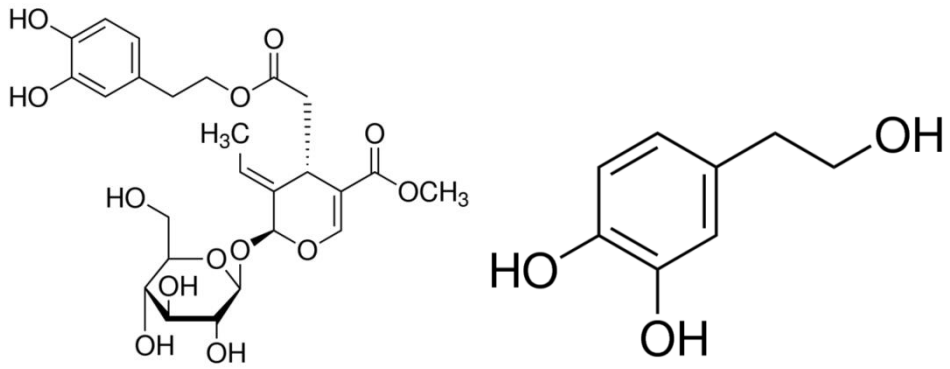


Figure 1.5 – Molecular Structures of Oleuropein and Hydroxytyrosol. Source: Sigma-Aldritch website [37]

Hydroxytyrosol has pharmacological properties and a powerful antioxidant activity, as powerful or even more than known antioxidants such as Vitamin E and Vitamin C. A similar antioxidant, tyrosol, does not have such a powerful antioxidant effect, thus suggesting that hydroxytyrosol's ring structure (orto-diphenolic) being essential for it. Its bioavailability, metabolism, and general health effects in humans have been heavily studied. Hydroxytyrosol is thought to be antagonistic against cancer and neurological and cardiovascular diseases, with oxidative stress effects on DNA thought to be mitigated by hydroxytyrosol. In fact, this and other antioxidants in olive oil appear to account for the lower incidence of some types of cancer in the Mediterranean region. Its effects on cardiovascular conditions could come from a

protective role towards low density lipoproteins and erythrocytes from oxidative stress, mitigating haemolysis and lipid peroxidation. [33]

But the potential health benefits of hydroxytyrosol do not stop here. It can also act as a metal chelating agent thanks to its ring structure. Effects on inflammation and platelet aggregation have also been described. Finally, hydroxytyrosol has also been suggested as having antimicrobial activity against respiratory and gastrointestinal infection agents such as *Haemophilus influenza*, *Salmonella typha*, *Vibrio parahaemolyticus*, *Vibrio cholera*, *Staphylococcus aureus* and *Mycoplasma pneumoniae*. In some cases, the minimum inhibitory concentrations are lower than those of some antibiotics like ampicillin. [33]

1.10.1.2 Possible applications

Since the appearance of alperujo as a new residue in the two-phase extraction method, there have been several studies with the purpose of enabling the valorisation of said residue. Many companies usually dispose of it in landfills where it can be toxic to the environment. [35] The most important possibilities include:

- Oil extraction
- Energy generation
- Fertilization
- High value compound extraction

Alperujo is usually treated with a second centrifugation to extract the residual oil. The resulting by-product of this second extraction is dried, and then subjected to chemical extraction with hexane in order to produce an extra yield of oil. This oil is known as olive-pomace oil (OPO). The OPO extraction industry uses a steam treatment to improve the solid-liquid separation, facilitating the drying for further extraction of OPO, a method already implemented at industrial scale. The treatment breaks cell wall structure, causes cellulose depolymerisation and hydrolysis of hemicellulosic material due to the generation of acids such as acetic and formic. Phenolic compounds (like hydroxytyrosol), fermentable simple sugars, oligo and polysaccharides and other high-added value compounds are released. The result is a significant solubilisation of the solid fraction in the liquid phase, and an enriched solid material. The oil is also concentrated in it, making it easier to recover it. [38]

But the recently discovered problems regarding the detection of dangerous polyaromatic hydrocarbons (PAH) in this oil has forced manufacturers to perform a further purification step, which greatly increases production costs. [38] The new resulting waste (dried and extracted alperujo, or “orujillo”) has a heat energy of around 400 kcal/kg [33], so it can be used for co-

generation of electrical and thermal power, but this method needs to be subsidized, and there is high production of ashes, which combined with the high quantity of residue produced every year, calls for different uses for the residue.

Alperujo could be used as a fertilizer due to its high organic content, including micronutrients. The residue is rich in phosphorous, but has a high C/N (carbon/nitrogen) proportion (nitrogen deficit), that could be corrected by nitrogen supplements, like manure. However, due to the residue's phytotoxicity, residue treatment is recommended, via bioremediation, for instance, with the goal of allowing degradation or polymerization of the phenolic compounds, reducing the residue's phytotoxicity. Alperujo can also be used as a nutrition source for ruminating animals, but a polyethylene glycol (PEG) supplement to block the phenolic compounds, and protein supplements to counter the low nitrogen content, should be used. [33]

Products of interest for the cosmetics, food, and pharmaceutical industries can be recovered from the residue. Powerful antioxidants like hydroxytyrosol, tyrosol and oleuropein, can be extracted. The potential of these, as mentioned before, is vast. The residue can be used for pectin production - pectin is used as a gelling, stabilizing and emulsifying agent in food industry. Alperujo can be mixed with thermoplastic polymers for container production. [33]

Finally, some producers are starting to separate the harder tissues present in alperujo due to its increasing potential as a flammable fuel, source of activated carbon, and to facilitate the composting of other organic materials. [33]

1.10.1.3 Alperujo's potential as a feedstock for biodiesel production

Due to its significant oil content, there is the possibility of using alperujo as a feedstock for biodiesel production. This is supported by the fact that OPO is not edible and has a low raw material price – the active market price of crude olive pomace oil varies from 0,6 to 0,7 €/L. Currently, most of the oil production is being forwarded for soap making purposes or for production of low quality olive oil blends. Much like olive oil, OPO's fatty acid composition varies greatly with the olive variant and growth conditions. The main fatty acid present in the OPO's triglycerides is Oleic Acid (C18:1), the preferred fatty acid for biodiesel production, and there are also significant amounts of Linoleic Acid (C18:2) and Palmitic Acid (C16:0). Also, OPO's free fatty acid content is usually around 0,2% (w/w) [39,40]

There are studies assessing the results of oil extraction and transesterification reactions. Hernández *et al.* (2013) [35] succeeded in extracting the residual oil using hexane and turning it into 94,7% biodiesel and 5,3% glycerol using methanol (in an alcohol:oil ratio of 1:2) as an acyl receptor and KOH as a catalyst. The resulting biodiesel was reported as conforming to ASTM

standards, and the oil-free alperujo was no longer dangerous for the environment. Muñoz *et al* (2014) [38] have reported yields of about 95% using a methanol:oil ratio of 6:1 using sodium hydroxide.

Using lipase from *Thermomyces lanuginosus* immobilized by covalent binding onto olive pomace, Yücel (2010) [21] has achieved a yield of 93%. It is worth noting that a three-step addition of methanol to avoid a strong enzyme inhibition was used, and also that the immobilized enzyme retained its activity for 10 consecutive batches of 24h reaction. In 2011, the same researcher optimized the parameters of the same method, reaching a yield of 93,73% yield by adding water (1% w/w), with the lipase (the same) also retaining activity for ten 24h long reacitons.[39]

Given the abundancy of Olive Pomace Oil, and the conclusions regarding biodiesel production with that oil, as well as the positive reviews of lipase and $scCO_2$ usage for biodiesel production, there seems to be some degree of potential to Alperujo as a feedstock for industrial scale biodiesel production, using a continuous reactor apparatus, and immobilized lipase. The usage of lipase has an important vantage, other than its catalytic power and environmental benefits: the oil has a high Free Fatty Acid content, which can be countered by lipases that can use it nonetheless to synthesise fatty acid alkyl esters.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Olive Pomace

The Olive Pomace used for this work was supplied by a local producer. As expected, it is a very humid, brown paste.

Figure 2.1 – Olive Pomace as was supplied

2.1.2 Solvents, Reagents and Standards

Table 2.1 – All the various solvents, reagents and standards used for this work

Material	Brand
Acetic Acid	Carlo Erba
Acetyl Chloride	Sigma
Acetonitrile	Sigma-Aldrich
Arabinose	Fluka
Carbon Dioxide	Air Liquide
Caffeic Acid	Sigma-Aldritch
Diethyl Ether	Sigma-Aldritch
Ethanol	Carlo Erba
Ferulic Acid	Sigma-Aldritch
Folin-Ciocalteu Reagent	Merck
Fructose	Sigma-Aldritch
Fucose	Sigma-Aldritch
Galactose	Sigma-Aldritch
Gallic Acid	Sigma-Aldritch
Glucose	Sigma-Aldritch
Hydroxytyrosol	Sigma-Aldritch
Mannose	Fluka
Methanol	Sigma-Aldritch
Methyl Heptadecanoate	Fluka
n-Heptane	Sigma-Aldritch
n-Hexane	Carlo Erba
Oleuropein	Sigma-Aldritch
p-Coumaric Acid	Sigma-Aldritch
Petroleum Ether	Sigma-Aldritch
Phenol	Sigma-Aldritch
Phenolphthalein	Home-made
Potassium Hydroxide	Pronalab
Quercetin	Sigma-Aldritch
Rhamnose	Fluka
Sodium Carbonate	Merck
Sodium Hydroxide	EKA Chemicals
Sodium Phosphate	Sigma-Aldritch
Sodium Sulfate	Carlo Erba
Sucrose	Sigma-Aldritch
Sulfuric Acid	Carlo Erba
Trichloroacetic acid	Home-made
Tyrosol	Sigma-Aldritch
Xylose	Merck

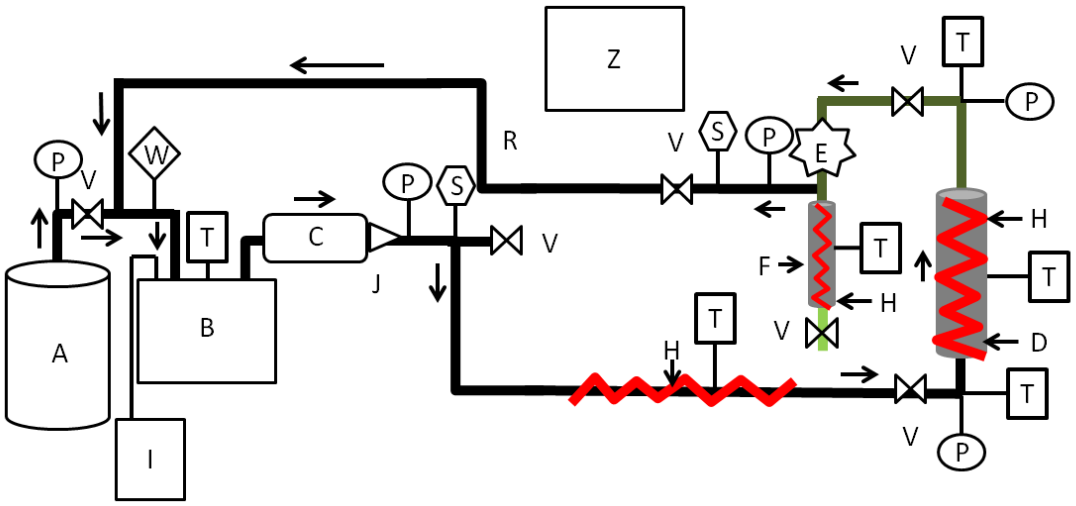
2.1.3 Enzymes

For enzymatic transesterification purposes, the lipase used was Lipozyme, an enzyme immobilized from *Mucor miehei*, produced by Sigma-Aldritch.

2.1.4 Supercritical Carbon Dioxide Apparatus

The apparatus used for all $scCO_2$ related experiments will be described in the following chapters. The apparatus for biodiesel production is the same as the one for oil extraction, save for a modification that will be detailed later on.

2.1.4.1 Supercritical Carbon Dioxide Apparatus for Oil Extraction



Main Circuit:
A – CO_2 Canister
B – Cold Bath
C – Liquid Pump
D – Reactor
E – BPR
F – Separator

P – Manometer
V – Valve
W – Flow Rate Indicator
I – Cryostat
T – Thermocouple
J – Check Valve
S – Safety Valve
H – Heating Stripe
R – CO_2 Recycling Circuit
Z – Master Control Unit

CO_2
CO_2 + Oil
Oil

Figure 2.2 – Schematic of the supercritical CO_2 extraction apparatus

The CO_2 (whose flow direction is represented by the arrows), after leaving the canister (A), is channeled into a cold bath (B) – water with ethylene glycol acting as antifreeze – cooled by cryostat (I) (Julabo F25), where it is cooled into liquid form. In this region, the carbon dioxide is kept at a pressure at around 6 MPa. As a liquid, it can be pumped by a compressed air powered liquid pump (C) (Williams V Series Metering Pump). As it is pumped, it goes through a check valve (J) and then reaches an area where the tubing is covered in a heating stripe connected to a thermocouple (H and T). In there, it will be heated and reach supercritical conditions. Between the check valve (J) and the Back Pressure Regulator, or BPR, (E) the pressure is kept at a much higher value – 30 MPa or higher – which is the actual function of the BPR.

The CO_2 reaches the reactor (D) (HiP TOC 7-20-G Reactor; with an inner diameter of 2,5 cm and a height of 51,5 cm, for a total inner volume of 252 mL), where the olive pomace has been placed – with free volume taken by glass spheres and the extremities having cotton. This is where extraction takes place, and this region is kept at a certain temperature by another heating stripe connected to a thermocouple – between 313 and 348 K, depending on desire conditions. It should be noted that the thermocouples not connected to heating stripes (before and after the reactor) were used for measuring purposes. The CO_2 and oil mixture then leave the reactor and finally go past the BPR, after which pressure returns to the approximately 6 MPa value. The carbon dioxide is no longer at supercritical conditions (instead, it cools greatly, existing as a gas and some solid) and cannot solubilize the oil, and as such the separator (F) have the oil deposited in its bottom where it can be extracted easily, just by opening the valve below the separator. While some carbon dioxide is lost by this procedure, most goes through the recycling circuit (R), where it returns into the pre-cryostat region.

The flow rate of carbon dioxide is measured by a high pressure flowmeter (RHEONIK RHM 007 GTM) (W) and controlled by handling the pump's controls and slight adjustments of the BPR.

For safety purposes, there are two safety valves (S), one after the pump and another just after the BPR and the separator, and some of the pressure indicators are connected to a digital display in a master control unit that automatically shuts down the pump if the pressure goes beyond a programmed value.

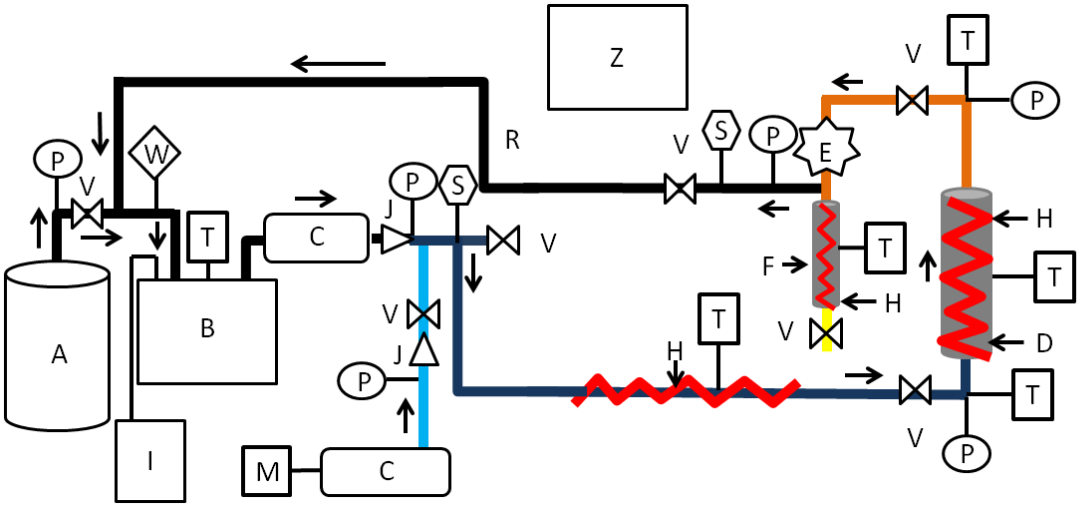
The oil was collected over time, and extraction curves were plotted. Based on existing works regarding lipid extraction with supercritical carbon dioxide, [41,42] two main parameters were calculated using the data: the oil yield and the oil loading.

The oil yield is calculated by dividing the total mass of oil obtained in the oil extraction by the mass of olive pomace introduced in the extraction reactor (g oil/100 g pomace).

The oil loading is the ratio of extracted oil versus the amount of carbon dioxide (g oil extracted per kg CO_2). It is calculated from the slope of the extraction curve, at the first period of extraction where saturation conditions of CO_2 prevail.

The oil was also used for fatty acid profile analysis, unsaponifiable matter content determination, and phenolic content determination.

2.1.4.2 Supercritical Carbon Dioxide Apparatus for Enzymatic Transesterification



Main Circuit:
A – CO_2 Canister
B – Cold Bath
C – Liquid Pump
D – Reactor
E – BPR
F – Separator

P – Manometer
V – Valve
W – Flow Rate Indicator
I – Cryostat
T – Thermocouple
J – Check Valve
M – Methanol
S – Safety Valve
H – Heating Stripe
R – CO_2 Recycling Circuit
Z – Master Control Unit

Figure 2.3 – Schematic of the supercritical CO2 transesterification apparatus

The apparatus for enzymatic transesterification is mostly similar to the extraction apparatus, with a number of changes. To integrate both oil extraction and biodiesel production, the reactor now contains both residue and enzyme, with the CO_2 passing through them in that order. Before the reactor, a small electricity powered pump (Gilson 305 Pump) is injecting methanol (M) into the main tubing with the $scCO_2$. This way, simultaneous oil extraction, mixture with methanol, and enzymatic transesterification occurs, integrating all processes. In a way, this simulates the existence of two reactors, one with alperujo where extraction occurs, and a second one with the enzyme to where the oil is channeled.

The flow rates of $scCO_2$ and methanol are controlled in order to achieve the desired methanol:oil molar ratios and enough $scCO_2$ to maximize oil extraction. This is done after calculation of the oil loading in $scCO_2$ (how much oil can be expected to be obtained with a certain amount of CO_2) and the average molar mass of the triglycerides in the oil. After the reaction, the scCO2 (and biodiesel) goes to the separator, where recovery of the products occurs just like in the extraction apparatus.

While the reaction can be carried out with ethanol (resulting FAEE instead of FAME), methanol was selected because it is cheaper, an important parameter when speculating on large-scale production processes.

The product that is deposited in the separator was collected over time and analyzed for FAME purity.

Figure 2.4 – CO_2 container, marking the beginning of the apparatus

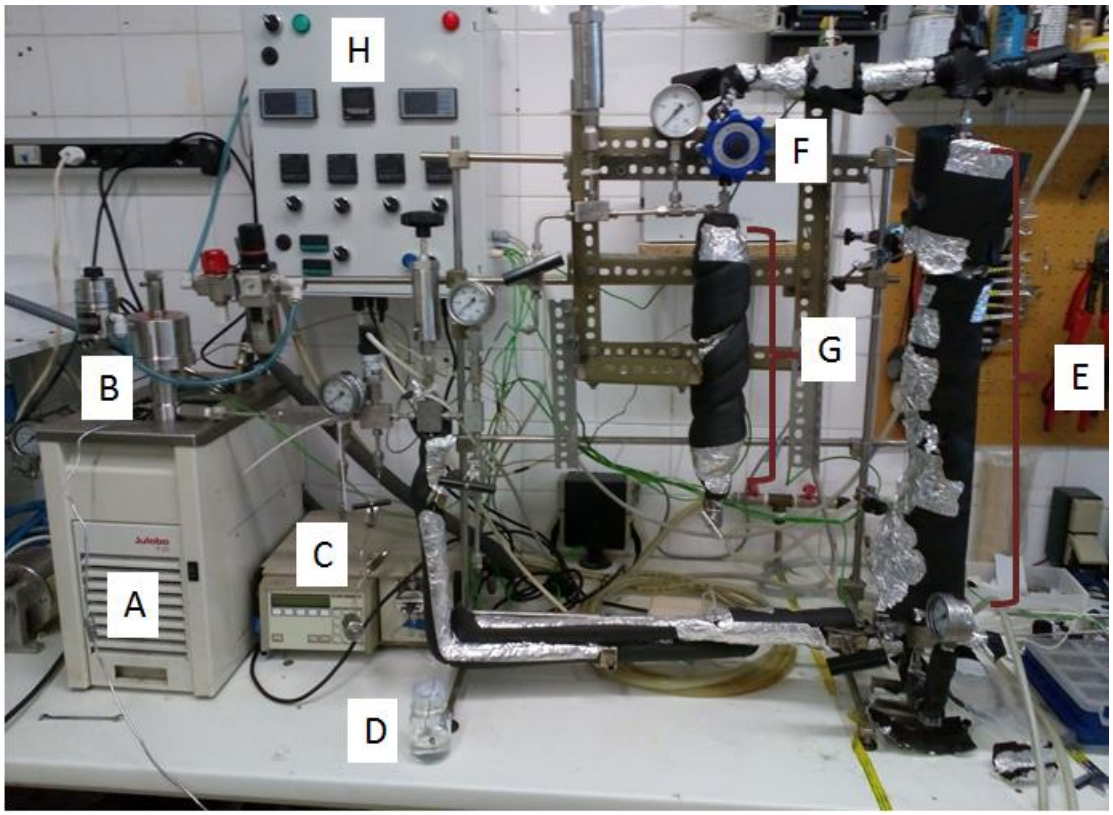


Figure 2.5 – Photo of the Supercritical CO_2 installation. A – Cryostat; B – Liquid Pump (CO_2); C – Liquid Pump (Methanol); D – Methanol; E – Extraction/Reaction Reactor (covered in insulating material); F – BPR (Back Pressure Regulator); G – Separator; H – Master Control Unit

2.2 Methods

2.2.1 Residue Treatment

2.2.1.1 Drying Pre-Treatment

Two methods to remove the water from the olive pomace were used, namely oven and freeze drying. Before and after the drying is complete, the residue was weighted for water content determination purposes.

Oven-drying: A large tray filled with residue was placed in an oven at a temperature of 353 K. With certain intervals (a few hours) the oven was opened and the residue was lightly swirled. After 2-3 days, the residue is removed and treated using a blender, and then placed again in the oven for a few more hours. This residue is then deemed “oven-dried”. This residue was used only for oil or phenol related studies.

Freeze-drying: A large balloon flask was filled with residue, and the freeze-drying was done with liquid nitrogen and then exposure to vacuum in a freeze dryer (B. Braun Biotech International Christ Alpha 1-4). This “freeze-dried” residue was used for all kinds of analytical studies, except for $scCO_2$ related experiments.

Figure 2.6 - Oven-dried (left) and Freeze-dried (right) Olive Pomace

2.2.1.2 Homogenization of Dry Olive Pomace

Due to the blender's inability to fully homogenize the residue, a fractionation was performed to some of the residue, using steel sieves (Endecotts Laboratory Test Sieves) with pore diameters of 710, 1400, 2000 and 2360 µm. The fractions were stored separately.

2.2.2 Extractions

2.2.2.1 Lipid Extraction from Olive Pomace

Soxhlet extractions were performed: 2g of pomace were placed in a packet made in filter paper, which was placed in a Soxhlet apparatus. The extraction was made using hexane under heating and lasted 4 hours. The hexane was then evaporated from the resulting oil using nitrogen.

2.2.2.2 Phenol Extraction from Olive Pomace

The phenols in the residue were isolated via a method termed Hydro-Alcoholic Extraction, based on the methodology used by Silva *et al* (2015): [43] 2g sample of residue were mixed with 40 ml of water:ethanol mixture (75:25 v/v) at 323 K for 18h, with constant magnetic agitation (150 rpm).

For preparation for analysis the mixture was filtered and extracts were obtained with 5 ml of diethyl ether – three times, each step lasting 10 min. The organic phase was dried with sodium sulfate (Na_2SO_4) and nitrogen. Finally, 5ml of a 1:1 methanol/water solution was added to the dry residue and the solvent and salt were filtered.

2.2.2.3 Phenol Extraction from scCO2 extracted Olive Pomace Oil

A modified version of the protocol described by Houshia *et al.* (2014) [44] was implemented, with the purpose of reducing the amount of reagents and oil used. 1g of oil was dissolved in 25 ml of hexane, and the phenols were extracted using three 25 ml portions of 80% aqueous methanol.

2.2.2.4 Hydrolysis of Carbohydrate Polymers and Isolation of Sugar Monomers

Based on the article by Deng *et al.* (2011) [45], a serial procedure to characterize the carbohydrate fraction of dry alperujo was implemented. The method is meant to separate the carbohydrate fractions into two parts: Soluble (monomers and oligomers, soluble in water/alcohol mixtures) and Insoluble (complex carbohydrates like cellulose and other structural sugars). The method also allowed separation of lipids and lignin. Along the way, the protein and phenolic fractions of the residue were lost. The method was composed of the following steps:

Soxhlet extraction: 2g of pomace were placed in a Soxhlet apparatus, with the extraction being performed for 4 hours using hexane and heating. The hexane was then evaporated from the oil using nitrogen.

Extraction of soluble sugars: using 40ml of 80% (v/v) ethanol:water solution, the sugar monomers and oligomers were extracted from 0,800 g of solid (the leftover from the Soxhlet extraction). The mixture went through an ultrasonic treatment for 15 minutes and was centrifuged (Beckman Coulter Avanti Centrifuge J-26 XP), with the supernatant being removed. The process was repeated three times, in duplicate – total of 1,6g of post-Soxhlet residue used. The supernatants were mixed and had their ethanol content dried using a rotary evaporator apparatus, and finally water was added to a final volume of 104 mL. A part of the solution were separated to undergo an acid treatment – 1mL of 72% H_2SO_4 were added to 28mL of the solution, and was left in an oil (Baysilone M350) bath at 393 K for 1h with constant magnetic agitation (150 rpm), in order to hydrolyze small carbohydrate oligomers into monomers. The solid precipitate was dried for the next step.

Acid hydrolysis of insoluble sugars – based on a protocol by Sluiter *et al* (2004)[46]: In a Schott flask, 0,3g sample of dried residue were mixed with 3mL of a 72% H_2SO_4 solution. The mixture was incubated with magnetic agitation (150 rpm) for 1h at 303 K, after which, the mixture was diluted to 4% (H_2SO_4) with 84 mL of water, and incubated for 1h, with agitation, in an oil (Baysilone M350) bath at 393 K. The remaining solid was dried for the next step.

Protein analysis: quantification of the remaining protein content allows subtraction of it from the residue's remaining mass. After also subtracting the ash content, the final mass quantity corresponds to Lignin.

The liquid fractions (Soluble Sugars and hydrolyzed "Insoluble Sugars") were analyzed via the Phenol-Sulphuric Method and HPLC methods as explained in Chapters 2.2.3.4 and 2.2.3.5, respectively.

2.2.3 Analytical Processes

2.2.3.1 Determination of Total Solids and Water Content

Based on a protocol by Sluiter *et al.* (2008) [47], a sample of the residue was placed in an oven at 373 K for four hours. Then, the sample was removed was allowed to cool to room temperature before being weighted to the nearest 0,1mg. The sample was placed in the oven again and dried to constant weight. Constant weight was defined as a ± 0,1% change in the weight of the sample after one hour. That is, the sample was dried until there were no more significant changes in its weight. That constant weight is the weight of the total solids, and the lost weight corresponds to the water content.

2.2.3.2 Determination of Protein Content

A sample of residue was subjected to the elemental analysis method CHNS performed by the Analytical Laboratory REQUIMTE – Chemistry and Technology Network Department of Chemistry, FCT, UNL. The protein content was derived from the N content found on dry biomass, using a conversion factor of 6,25.

2.2.3.3 Determination of Ash Content

2 g of dry residue were weighted into ceramic plates and put in a heater at 848 K for 4 hours. The remaining residue was weighted, and compared to the initial mass.

2.2.3.4 Total Carbohydrate Quantification – Phenol-Sulphuric Method

The total carbohydrate content in a given sample (aqueous solutions resulting from previously described hydrolysis and extractions) was quantified using a photometric method based on a modified version of a method by Masuko *et al* (2005). [48] On a test tube, a 500µl sample of dissolved sugars (post acid hydrolysis) received 1,5 ml of a 96% H_2SO_4 (Sulfuric Acid) solution and 300µl of a 5% phenol solution. The mixture was incubated in a dry bath (Accublock™ Digital Dry Bath) at 363 K for 5 min. After cooling the tubes to room temperature in a water bath, the absorbances at 490nm were measured with a spectrophotometer (DU®800 Spectrophotometer from Beckman Coulter, Brea, USA). The sugar concentrations

were determined in glucose equivalents using glucose standards – 10, 25, 50, 75, 100, 250, 500, 750 and 1000 mg/l - and mili-Q water blank.

2.2.3.5 HPLC Characterization and Quantification of Sugar Monomers

While total carbohydrate quantification was performed in the lab, the task of actually identifying them and quantify each monomer for each sample was seconded to the Analytical Laboratory REQUIMTE – Chemistry and Technology Network Department of Chemistry, FCT, UNL. This was done by HPLC analysis using lab-prepared standards of various sugar monomers – Glucose, Fructose, Xylose, Arabinose, Mannose, Fucose, Sucrose and Rhamnose, at 1g/L concentrations – in both Mili-Q Water and 4% H_2SO_4 (since there are samples dissolved in water, and others in diluted acid solutions).

2.2.3.6 Determination of Total Phenolic Content – Folin-Ciocalteu Method

The total phenolic content was determined using the Folin-Ciocalteu Method. [49] The calibration standards were five Gallic Acid solutions (50, 100, 150, 250 and 500 mg/l), and the blank was Mili-Q water. The samples were previously treated for protein precipitation: 800 µL of sample were mixed with 120 µL of 100% trichloroacetic acid (TCA) solution. After 5 minutes at 253 K and 15 minutes at 277 K, the solution was centrifuged (Heraeus Sepatech Biofuge 13) for 15 min at 12000g. The precipitate was discarded.

As for the Folin-Ciocalteu Method itself, to 20µl of sample/standard/blank, 1,58 ml of distilled water and 100µl of Folin-Ciocalteu reagent (mixture of phosphomolybdate and phosphotungstate) were added, and the mixture was stored at room temperature for 8 min. Then, 300µl of sodium carbonate solution (200g/L) were added and the mixture was incubated in a dry bath (Accublock™ Digital Dry Bath) at 313 K for 30 min.

The final solution's absorbance was measured at 750 nm (it should be 765nm but due to spectrophotometer limitation it was measured at 750nm) with a DU®800 Spectrophotometer from Beckman Coulter (Brea, USA). The concentrations of phenolic compounds were determined at mg/l GAE (gallic acid equivalent).

2.2.3.7 HPLC Quantification of Phenols

In order to identify and quantify individual phenolic compounds in the residue or oil based extracts, an analysis was performed with a HPLC Chromatograph from Thermo

Scientific: Finnigan Surveyor Autosampler Plus, Finnigan Surveyor LC Pump Plus. The column was a reverse-phase polymeric C_{18} column. Software used for data treatment was ChromQuest 5.0. The absorbance is measured at two different wavelengths: 280nm and 320nm with a Accela UV/Vis Detector. The mobile phase is a mixture of two eluents: a 2% acetic acid solution in Mili-Q water, and a mixture of (Mili-Q) water and acetonitrile (50:50) with 0.5% acetic acid (v/v). The injection volume was 5 µL and total run time was 30 min. For each compound being analyzed, a corresponding standard concentration curve was created.

The identification and quantification of oleuropein was done using a different mobile phase: a phosphate buffer solution (10mM) and acetonitrile, in a 80:20 (v/v) ratio, with a set pH value of 3. This method is based on a protocol by Al-Rimawi (2013).[50]

Quercetin identification and quantification was also done using a different mobile phase – a Methanol/Water/Phosphate solution (60:40:0,4 v/v) – based on a protocol by Zhishen *et al* (1999). [51]

2.2.3.8 Determination of the Fatty Acid Profile of extracted oils

For this objective, the Lepage & Roy method was implemented, which consists on the direct transesterification of oils, forming methyl esters that are then identified. Each methyl ester corresponds to the fatty acid from which it derives.

10-25 mg portions of oil were mixed with 2 mL of methanol:acetyl chloride 95:5 v/v solution and 0,1 mL of methyl heptadecanoate (internal standard; 10 mg/mL). The mixture was heated at 353-358 K for 1 hour in a water bath, isolated from light. The mixture was then cooled at room temperature and diluted with 1 mL of n-heptane and 1 mL of water, to facilitate phase separation. The upper phase with heptadecanoic was transferred to a cotton filter bed with a portion of anhydrous sodium sulfate to remove the water, and finally filtered. The final solution was analyzed via GC.

Peak identification was carried using known standards and the software Chrom-Card.

2.2.3.9 FAME Content Determination in Biodiesel Samples

The EN14103 method for methyl esters determination was implemented. It requires GC analysis with a programmable temperature vaporizing (PTV) injector and a wax column. The tested specifications stipulate that biodiesel should have a ester content greater than 96,5% m/m and linolenic acid methyl ester content lower than 12% m/m.

25 mg of oil extract was weighted in a 10 mL vial, then 500 µL of methyl heptadecanoate internal standard solution was added – 10mg/mL in heptane. The mixture was then injected and analyzed via GC.

2.2.3.10 Unsaponifiable Matter Quantification

A modified version of the AOCS Official Method (Ca 6a-40) was used. The modifications had the purpose of reducing the amount of reagents used in each analysis by half. The unsaponifiable matter was determined using the following formula:

$$Unsaponifiable\ Matter\ (\text{wt.}\%) = \frac{A - (B + C)}{sample\ mass} \times 100$$

Equation 2.1 – Determining the unsaponifiable matter content in oil

A 2,5g ± 0,1 mg sample of oil were weighted into a flask, where 15mL of 95% ethanol and 2,5mL of 50% (w/w) KOH were added. The mixture was boiled gently and refluxed for 1 hour. The mixture was transferred to a separatory funnel and the flask washed to the 20 mL mark with 95% ethanol, then with 20 mL of distilled water, and finally with 5mL of petroleum ether, all of which were added into the funnel.

After cooling to room temperature, 25mL of petroleum ether were added, and the mixture was shaken vigorously for 1 min, and both layers were left stationary for 10 minutes to be allowed to settle and become clear. The upper layer (petroleum ether) was removed, and the extraction repeated six times. All the petroleum ether fractions were combined in a flask, and washed several times with 25mL portions of 10% ethanol, with the alcohol layers being drawn off. This washing continued until there was no pink color in the wash solution after addition of one drop of phenolphthalein solution (over 3 extractions were needed to achieve this). The petroleum ether was evaporated with a rotary vapor apparatus, and the residue was weighted (value A).

The residue was dissolved in 25 mL of 95% ethanol at 323 K with previously neutralized phenolphthalein and the mixture was then titrated with 0,02M NaOH solution to the same final color. It was considered that 1mL of NaOH solution is needed to neutralize 0,0056g of oleic acid, so the free fatty acid amount (value B) was determined directly from the amount of NaOH solution needed to neutralize the acid. The same procedure was implemented without any oil for a reagent blank (value C).

CHAPTER 3: RESULTS AND DISCUSSION

3.1 General Composition of Olive Pomace

The first objective of the project was to determine the general composition of the olive pomace, specifically: water, lipid, protein, carbohydrate, lignin and ash contents. The various protocols for this objective are described in Chapter 2. After drying the residue (Chapter 2.2.1.1), the extractions of various compounds were made according to Chapters 2.2.2.1 (for lipids), 2.2.2.4 (for carbohydrates and lignin) and the analytical processes are described in chapters 2.2.3.1 to 2.2.3.4 – water, protein, ash and carbohydrate contents respectively.

The residue, in its provided state, has a calculated average of 70,84% +/- 0,02 of water, which is consistent with literature. [34] After the water was removed, the remaining mass (dry mass) is what was analysed in all posterior biomass analysis.

The basic composition of the dry residue was determined as follows:

Table 3.1– Basic composition of the dried olive pomace

Component	wt.%
Lipids	17,81 +/- 0,18
Protein	11,28 +/- 0,16
Ashes	3,74 +/- 0,05
Carbohydrates (Soluble)	4,19 +/- 1,71
Carbohydrates (Non Soluble)	7,90 +/- 0,05
Lignin	26,97 +/- 4,04

The results for lipids, protein and ash content are within the intervals set in literature [34] and were consistent and easily duplicated. On the other hand, the consistency of the results regarding the soluble carbohydrates and lignin was unexpectedly low. Even with the highest values of the intervals, the total sum was 77,54%. This could be due to the residue's non homogeneous form, or possible interferences with the sugar quantification protocol which leads to faulty sugar values. The soluble carbohydrate content value (4,19%) is in accordance with literature values [34] of 1-16% for water-soluble carbohydrates. But, the actual total sugar content is possibly higher than what was determined. Based on Table 1.4,[34] where the cellulose and hemicellulose content are stated to account for 40-65% of the residue's dry weight, a portion of the structural sugar content was possibly lost.

In order to complement the carbohydrate content analysis, the two extracts (Soluble and Non Soluble) were also analysed via HPLC (Chapter 2.2.3.5) for their sugar monomer profile. The results are summed by in the following figures.

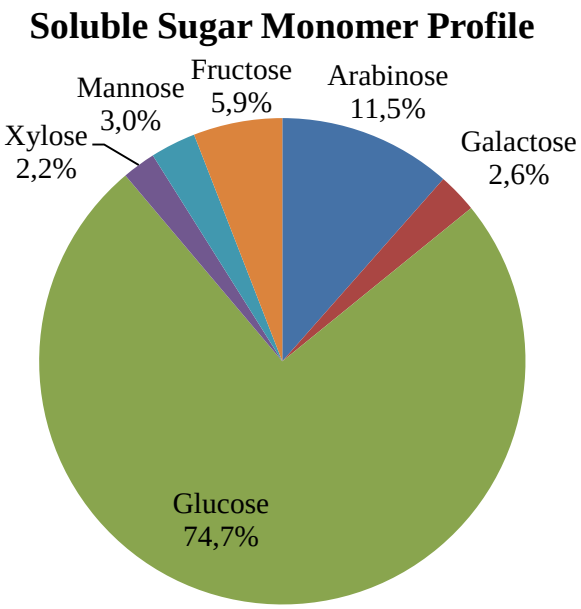


Figure 3.1 - Sugar Monomer Profile of the Soluble Sugar fraction

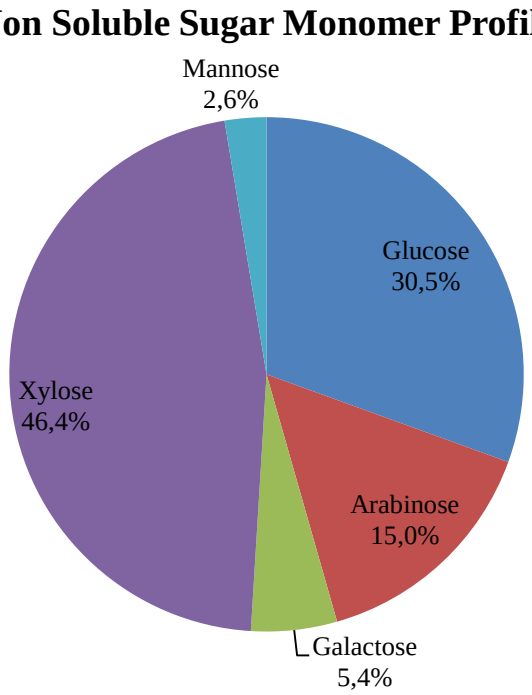


Figure 3.2 - Sugar Monomer Profile of the Non Soluble Sugar fraction

There are also other probable components that were not analysed, such as sterols, squalene and tocopherol. While present in olive oil, they most likely do not exist in this olive pomace in significant amounts to account for the missing biomass percentage (approximately

23%). The phenolic compounds that were quantified, and detailed in Chapter 3.6, were left out of the table, but are not a significant contributor – less than 0,1% of the dry mass, as will be shown later on – so the suggested compounds are possibly not significant enough as well.

3.2 Olive Pomace Oil Extraction via scCO$_2$

Using the apparatus and procedures described in Chapter 2.1.4.1, various extractions were performed under multiple variable conditions – temperature and pressure. The CO_2 flow rate and total CO_2 amounts used were also recorded to help calculate the oil's loading in CO_2.

The extraction curves (see Figure 3.3) were drawn by plotting the oil yield (wt.%) against the mass ratio between the CO_2 amount used and the total mass of olive pomace (OP) inserted in the reactor (mCO_2/mop), instead of the extraction time. The reason for this was to allow comparison between extraction curves made at different operating conditions – CO_2 flow rates and residue masses, which could not be perfectly constant for every extraction.

Extractions took between 1 and 3 hours, with an average of 90 minutes. The CO_2 flow rates was kept stable for each individual extraction, and varied between 11 and 19 g/min. As each extraction curve is shown, the correspondent CO_2 flow rate will be stated in the figure's captions.

The amount of residue used was approximately 100g for all experiments. Only oven-dried residue was used, since freeze-drying enough residue for the multiple extractions performed would be cost and time ineffective because of the low amounts that can be dried each time. Since this is a simulation of a large scale process, the cost of a method when applied to larger-than-lab scales should be considered.

3.2.1 The Issue of Homogenization

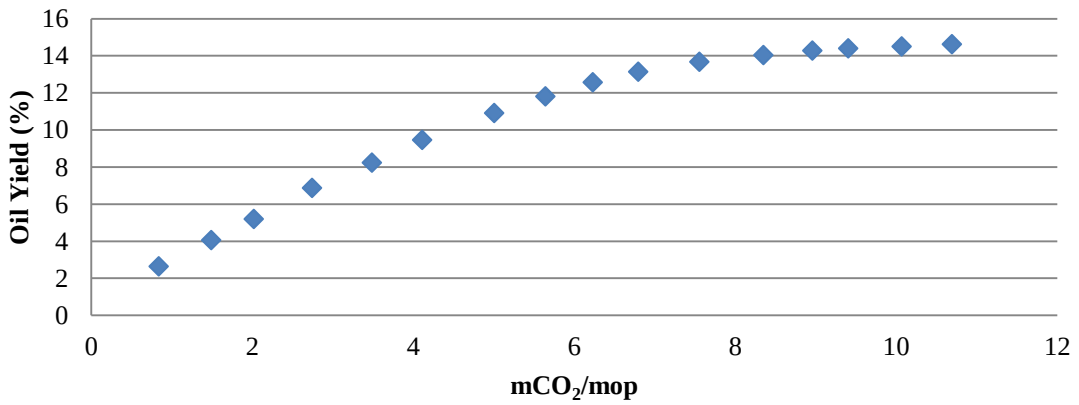


Figure 3.3 – Example of an extraction curve, taking place with P/T conditions of 50 MPa and 323 K, and at a CO_2 flow rate of 15 g/min

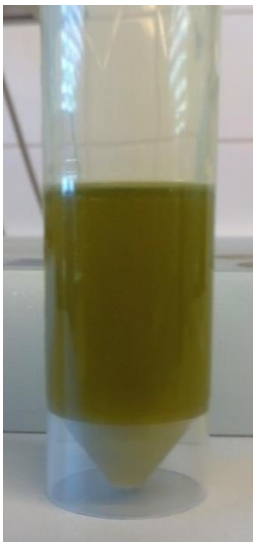


Figure 3.4 – Olive Pomace Oil, extracted via scCO2

Because the residue is not fully homogenised, since it was provided to the lab in its original, untreated state, lack of uniformity in particle size could be a serious issue with this residue. Smaller particles, made mainly of pulp, may have more oil content than the seeds. Using the original residue as it came could be less efficient than concentrating the pulp with a pre-fractionation of the olive pomace and removing the seeds. Thus, as per described in Chapter 2.2.1.2, the original olive pomace was fractionated with the help of several sieves of different sizes.

Figure 3.5 – Various fractions of dry olive pomace, classified according to approximate size. From left to right: <710 µm; 710-1400 µm, 1400-2000 µm, 2000-2360 µm, >2360 µm

The most important fraction collected was of those particles smaller than the pores with a diameter of 710 µm. This fine, dark brown dust composed around 30% of the dry pomace, and as shown below, is much richer in oil than the rest of the pomace. Other fractions, in crescent order, had increasingly less pulp and more seeds, therefore, lower oil content. Large, condensed

pieces of pulp were also observed. Around 30% of the dry pomace had particles over 2000 µm of diameter. The biggest sieve (2360 µm of pore diameter) caught around 15%.

3.2.2 Effect of Particle Size on Extraction

In order to assess the influence of particle size on oil the extractions, a series of experiments were performed at similar operating conditions but different residue particle size fractions, and the results compared to the average, bulk residue. CO_2 flow rate varied between 13-16 g/min throughout these extractions

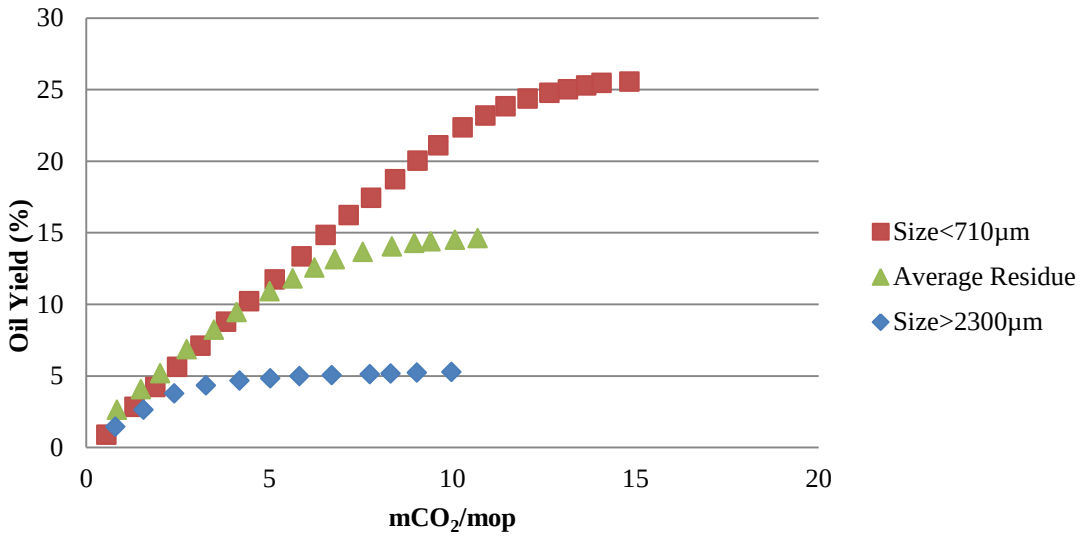


Figure 3.6 – Several extraction curves (50 MPa; 323 K) showing the influence of particle size. CO_2 flow rates for each extraction: Size<710µm – 13 g/min; Average Residue – 15 g/min; Size >2300µm – 18 g/min

The largest particles had a very low oil content (5,3 wt.%), most likely due to the carbon dioxide being unable to diffuse inside the large particles, and also because of the olive nuts not having as much oil as the pulp. The following table and analysis focuses on the finest residue compared to the bulk residue.

Table 3.2 – Yield ($g_{oil}/g_{dry\ residue}$) comparisons between fractions (fine vs bulk residue) and extraction methods (scCO2 vs hexane)

Residue	Bulk Residue	<710µm Fraction
$ScCO_2$ Yield (%)	14,7 +/- 1,0	25,5 +/- 0,8
Soxhlet Yield (%)	17,8 +/- 0,2	28,9 +/- 0,8
$ScCO_2$ Efficiency	82,6 +/- 6,3	88,4 +/- 4,8

When a homogenised, pulp rich, small particle size fraction was used, much better oil yields were achieved, and more mass of CO_2 was needed to complete the extraction process. The fine pulp particles are much richer in oil than the rest of the residue; moreover, the finer the particles, the easier carbon dioxide diffuses inside them, leading to a more complete extraction.

The oil yields from the $scCO_2$ based extraction are not as high as the Soxhlet extraction yields (hexane), which is in accordance with literature.[25] However, while Lucas *et al.* (2002) described a $ScCO_2$/Soxhlet ratio of 80% with optimal conditions (30 MPa, 328 K), in this work, the ratio was close to 90% (50 MPa, 323K). This is most likely due to the pressure (50 MPa) involved as it will be described later.

Since the oil yield using hexane for extraction is higher than even the optimized extraction with $scCO_2$, that means some oil is left in the residue after the extraction with carbon dioxide. This is confirmed by performing a Soxhlet extraction on the residue that is left after the $scCO_2$ extraction. This was performed for the fine residue (<710µm), and the post-extraction residue had an average amount of 4,9 +/- 0,4 % of oil in it.

Figure 3.7 – Pre-extraction olive pomace and post-extraction olive pomace (left and right, respectively; oven-dried fine residue fraction)

3.2.3 Pressure and Temperature Effects on Extractions

In order to assess the influence of pressure and temperature on the $scCO_2$ extraction of olive pomace oil, several experiments were performed – using the finest residue (<710µm) fraction – at different operating conditions and their extraction curves compared. The objective was to assess the best conditions for extracting the oil from olive pomace by $scCO_2$.

Both pressure and temperature have a significant influence on the extraction yield. E.g., higher pressures of extraction lead to better oil yields and also to lower CO_2/residue mass ratios. But, an important factor to consider is the energy consumption on the process. Higher pressures require more energy consumption, through the gas pump. At a certain point, there are diminishing returns by increasing the pressure. As such, several conditions must be tested in order to optimize the process.

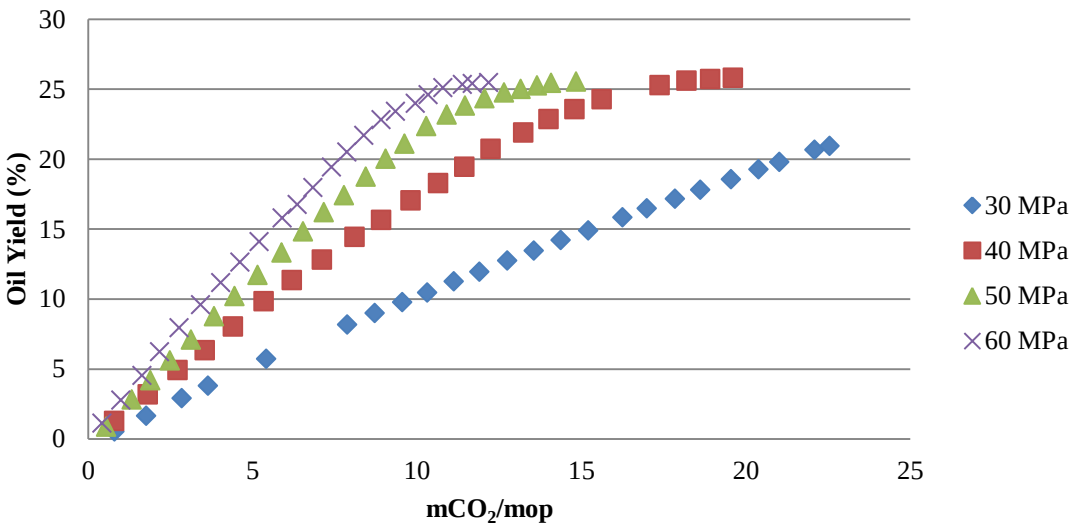


Figure 3.8 – Extraction curves with a constant temperature of 323 K. CO_2 flow rates for each extraction: 30 MPa – 19 g/min; 40 MPa – 18 g/min; 50 MPa – 13 g/min; 60 MPa – 11 g/min.

According to Figure 3.8, at a constant temperature of 323K, an increase in pressure (30 to 60 MPa) led to better oil yields, as expected. Increasing the extraction pressure leads to higher densities of carbon dioxide, which increases the oil's solubility. But as the figure shows, the increase in the extraction pressures led to increasingly less gains in the oil yield.

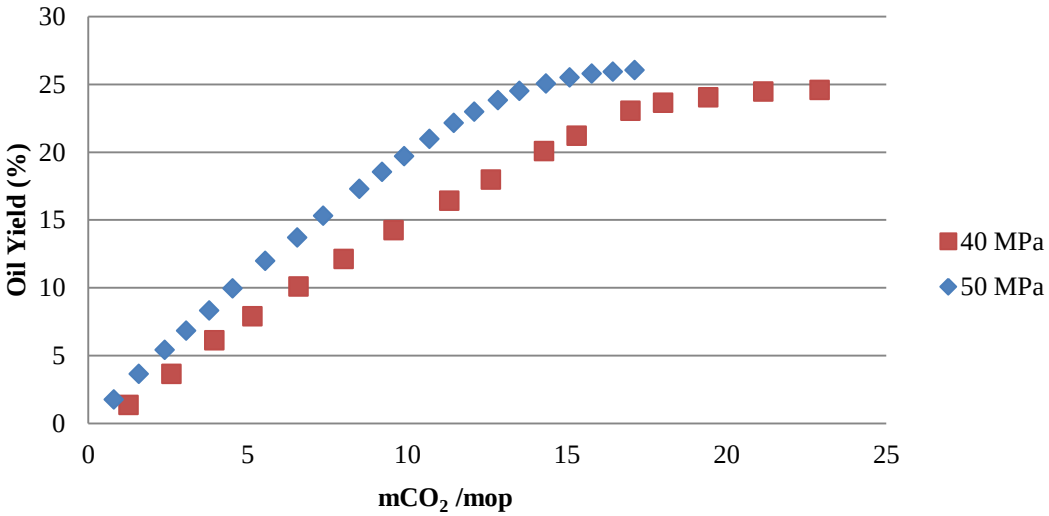


Figure 3.9 – Extraction curves with a constant temperature of 313 K. CO_2 flow rates for each extraction: 40 MPa – 16 g/min; 50 MPa – 17 g/min

The same effect of the increasing pressure was observed at 313 K.

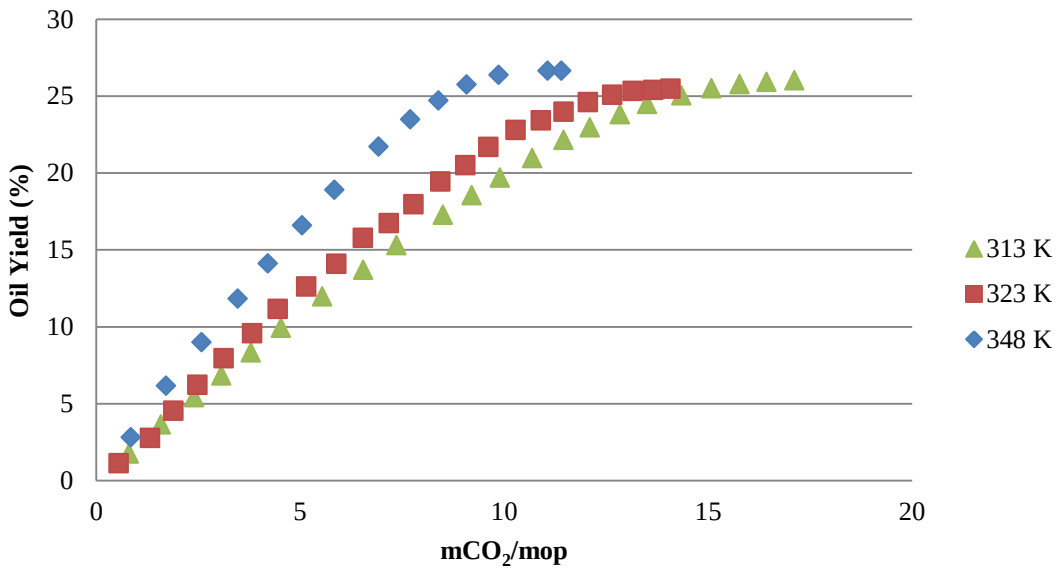


Figure 3.10 – Extraction curves with a constant pressure of 50 MPa. CO_2 flow rates for each extraction: 313 K – 17 g/min; 323 K – 13 g/min; 348 K – 18 g/min.

Figure 3.10 shows the effect of the temperature on the yield of extraction at a constant pressure of 50 MPa. Higher temperatures of extraction led to higher oil yields.

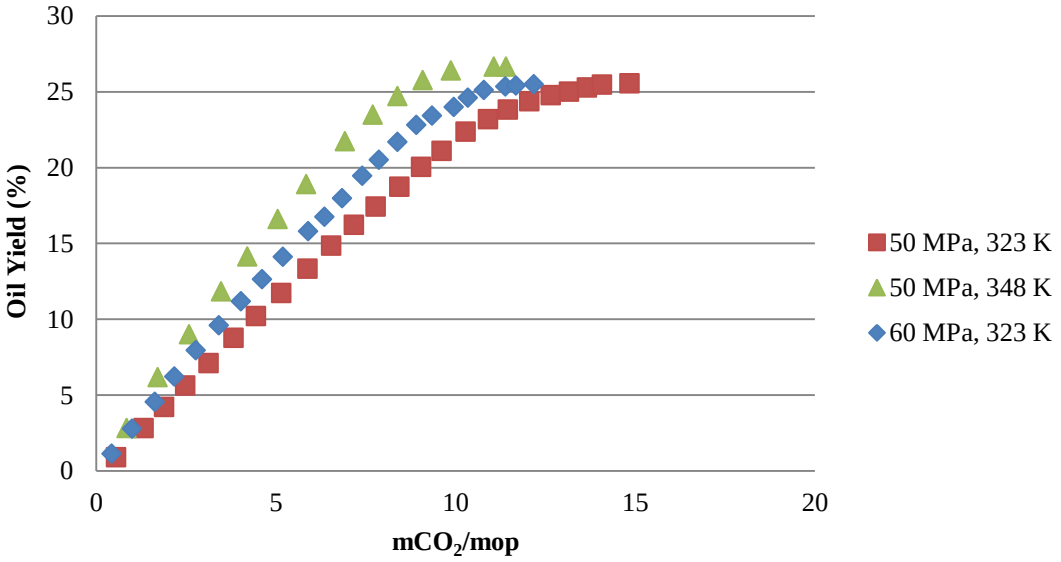


Figure 3.11 – The three extraction curves for the three highest operating conditions. CO_2 flow rates for each extraction: 50 MPa, 323 K – 13 g/min; 50 MPa, 348 K – 18g/min; 60 MPa, 323 K – 11 g/min.

Figure 3.11 shows that out of all the tested operating conditions, the best conditions for oil extraction were established to be 50 MPa and 348 K.

3.2.4 Calculating Oil Loading for Olive Pomace Oil Supercritical Extraction

While the curves themselves are an indicator of how well the oil is extracted, an important value that should be determined from the extraction curves is the oil loading in the supercritical carbon dioxide, calculated in g oil/kg CO_2. For demonstration purposes, the following figure shows the slope from a portion of an extraction curve.

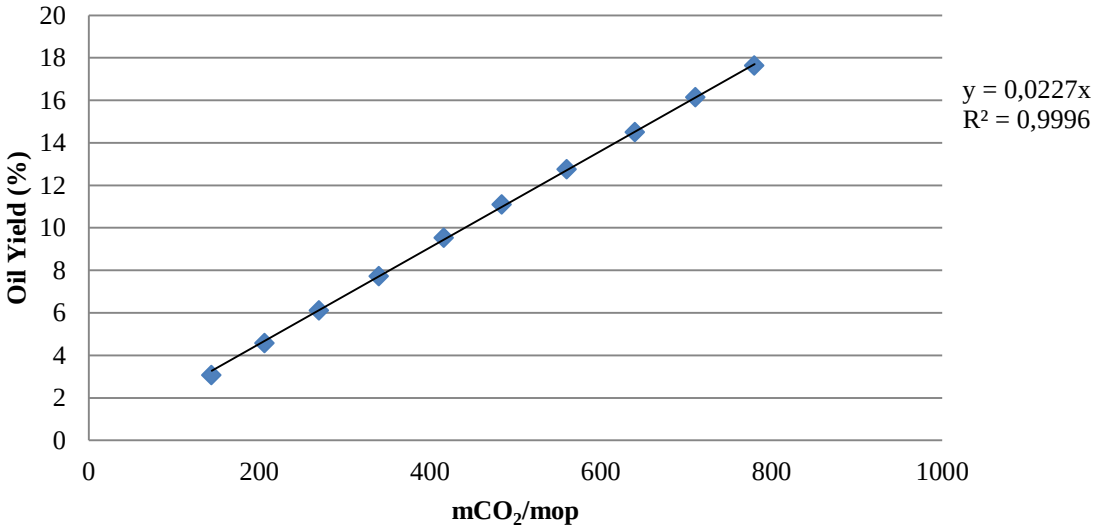


Figure 3.12 – Example of a line for oil loading calculation for an extraction at 50 MPa and 323 K

From the slope (0,0227 g oil/g CO_2) it is possible to calculate an oil loading of 22,7 g oil/kg CO_2 for operating conditions of 50 MPa and 323 K.

Table 3.3 - Calculated oil loadings for the pressure and temperature operating conditions used. Loadings are in g oil/kg CO_2

P/T (MPa/K)	313 K	323 K	348 K
30 MPa	-	9,70	-
40 MPa	14,70	18,10	-
50 MPa	21,90	22,70	32,60
60 MPa	-	26,40	-

The calculated oil loading values reflect what had already been previously observed earlier during the analysis of the extraction curves. Using the same temperature but increasing pressure conditions, the oil loading increases, with the same happening for the same pressure but increasing temperature conditions.

The greatest oil loading value calculated was 32,60 g oil/kg CO_2, for operating conditions of 50 MPa and 348 K, which is in accordance with Figure 3.11, where those conditions were already concluded to be the most optimal among the conditions used. That value is 3,4 times greater than the lowest obtained oil loading value – 9,70 g oil/kg CO_2 – which corresponds to the least optimal conditions as already concluded in Figure 3.8 – 30 MPa and 323 K.

The calculated loadings were converted into kg oil/m^3 CO_2 and compared to the results from two mathematical correlations of solubility data of vegetable oils in $scCO_2$, one from Valle *et al* (1988) [52] and another from Sovová *et al* (2001)[53], both referenced in a review by Valle *et al* (2006). [42] The two following equations predict vegetable oil solubility in $scCO_2$ as the product of a density-dependent term, and a temperature-dependent term.

$$c_{sat} = \rho^{10,742} \exp(-40,361 - \frac{18708}{T} + \frac{2186840}{T^2})$$

Equation 3.1 - Correlation of solubility data of vegetable oil in supercritical carbon dioxide by Valle *et al* (1988) [52] . c_{sat} is expressed in kg oil/m^3 CO_2; ρ is the CO_2 density in kg/dm^3; T is absolute temperature.

Equation 3.1 was validated by the authors with data from the solubility of soybean oil, sunflower seed oil, cottonseed oil and corn oil, for absolute temperatures from 293 to 353 K, pressures between 15,2 MPa to 89,2 MPa and oil solubilities below 100 kg oil/m^3 CO_2.[52]

$$c_{sat} = \rho^{1,4+0,0048\rho-0,000002\rho^2} \exp(-10,14 - \frac{5000}{T})$$

Equation 3.2 – Correlation of solubility data of vegetable oil in supercritical carbon dioxide by Sovová *et al* (2001) [53]. c_{sat} is expressed in kg oil/m^3 CO_2; ρ is the CO_2 density in kg/m^3; T is absolute temperature.

Equation 3.2 was validated by the authors for refined blackcurrant seed oil, [53] and can be used to predict oil solubility in $scCO_2$ for pressures up to 60 MPa, a temperature range of 293 to 373 K and oil solubilities greater than 0,5 kg oil/m^3 CO_2, as stated by Valle *et al* (2006).[42]

The experimentally calculated oil loadings and the oil solubility predictions based on Equations 3.1 and 3.2 for the various pressure and temperatures conditions used in this work are shown in the following table.

Table 3.4 – Calculated oil loadings (kg oil/m^3 CO_2) for the various P/T conditions tested. In brackets are the calculated values of oil solubility (Equations 3.1 and 3.2, respectively).

P/T (MPa/K)	313 K	323 K	348 K
30 MPa	-	8,44 (6,82/6,6)	-
40 MPa	14,05 (11,44/11,59)	16,71 (12,84/13,01)	-
50 MPa	21,71 (16,88/16,78)	21,85 (20,06/20,36)	29,02 (30,47/26,07)
60 MPa	-	26,23 (28,33/28,21)	-

The predicted solubility values for both models had differences below 1 kg oil/m^3 CO_2, except in the 50 MPa/348 K scenario, where the difference was greater than 3 kg oil/m^3 CO_2. This is not unexpected, as Valle *et al* (2006) [42] described how the two models are in excellent agreement at temperatures between 303,45 and 336,25 K, and the aforementioned scenario involved a temperature of 348 K, at which point the first model delivered higher values than the second one. According to Valle *et al* (2006) [42] and Sovová *et al* (2001) [53], the second model is better suited for oils under the conditions used in this work – the authors of that correlation concluded that the solubility of triglycerides in scCO_2 is a strong function of the their molecular weight, and took into account that most plant oils are triacylglycerols of fatty acids with 18 carbon atoms, like stearic and oleic acids. Because of this, the calculated oil loading at 50 MPa and 348 K (29,02 kg oil/m^3 CO_2) was compared only with the predicted value for the second model (26,07 kg oil/m^3 CO_2).

The determined oil loading values were greater than the predicted solubility, which is not unexpected, since the mathematical correlations predict oil solubility, not loading. The difference was greater in the 50 MPa/313 K scenario, and the 50 MPa/348 K scenario for the second model – the first model delivered a higher oil solubility value than the calculated oil loading, which is possibly related to the previously described discrepancy between the two prediction models.

3.3 Olive Pomace Oil Properties

After oil extraction, the collected oil samples were analysed for the following parameters: Fatty Acid Profile and Unsaponifiable Matter. Additionally, the oil phenolic content was determined, which will be discussed further in Chapter 3.6.

3.3.1 Fatty Acid Profile of Olive Pomace Oil

The fatty acid profile, that is, the fatty acids that exist in the triglycerides (and in other glycerides or free form) of the extracted oil, was determined via Gas Chromatography, via the method described in Chapter 2.2.3.8.

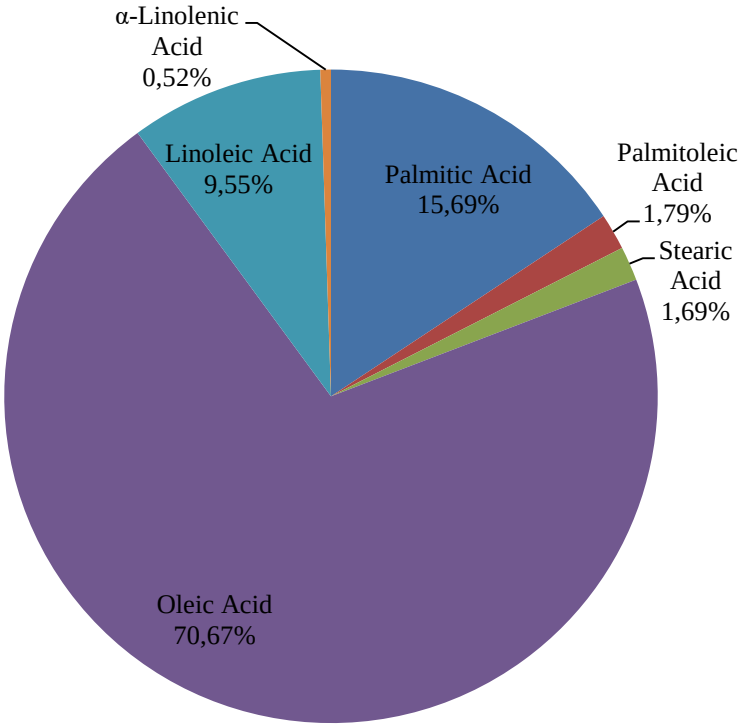


Figure 3.13 – Average fatty acid profile of the extracted olive pomace oil, in % (m/m)

The determined profile is in accordance with literature [40] that states that Oleic Acid is by far the most abundant one, followed by Palmitic and Linoleic Acids. Remaining detected acids (Stearic, Palmitoleic and α-Linolenic) came in very low concentrations. According to literature, other acids can exist in very low amounts (less than 1% wt.%), like Tetradecanoic or Icosanoic Acids but were not detected in this work.

3.3.2 Unsaponifiable Content of Olive Pomace Oil

Because it is not convertible in biodiesel, unsaponifiable matter content was determined, using the procedure described in Chapter 2.2.3.10.

Unsaponifiable matter content was determined as being 1,03 +/- 0,26 wt.%. It is in accordance with literature, which stipulates a value under 3%.[54]

3.4 Biodiesel Production from Olive Pomace Oil

Another objective in this work is biodiesel production using olive pomace, as a proof of concept for combination of olive pomace oil extraction, enzymatic transesterification of the oil, and recovery of the biodiesel in an integrated process using supercritical carbon dioxide. As shown in Chapter 1.10.1, there is already evidence that olive pomace, an abundant agricultural waste, can be used as a feedstock for biodiesel production.

3.4.1 Main Reaction Parameters

In Chapter 2.1.4.2 the apparatus used for biodiesel production is described. There are two main parameters that had to be calculated and chosen before any experiments, i.e., the residence time (t_r) and the oil:methanol molar ratio. Both parameters will determine the CO_2 and methanol flow rates to be used in the reaction experiments.

The residence time parameter refers to the time taken by the oil to pass through the enzymatic section of the reactor after being extracted from the pomace residue, since the enzyme is placed after the olive pomace residue, as per described in Chapter 2.1.4.2. The longer the time oil is exposed to the enzymatic bed, the higher the yield of conversion to biodiesel, provided enough methanol is supplied. The residence time can be calculated using the following equation:

$$t_r = \frac{\rho_{CO_2}}{Q_{CO_2}} * V_{enzyme\ in\ reactor}$$

Equation 3.3 – How to calculate Residence Time (t_r) from CO2 density (ρCO_2), flow rate (QCO_2) and a volume of enzyme ($V_{enzyme\ in\ reactor}$)

For a desirable residence time, the volume of enzyme required can be calculated, if we know the CO_2 density (dependent on the operating pressure and temperature conditions) and the

flow rates. Knowing that the density of the enzyme is 0,42 g/cm^3, it is possible to convert the volume of enzyme into a easily weighable mass.

The oil:methanol molar ratio, is the stoichiometric ratio between triglycerides and methanol molecules during the reaction. While 1:3 is the actual reaction ratio, a methanol excess is recommended to shift the reaction equilibrium towards biodiesel production. Yet, the presence of too much methanol can inhibit the enzyme, so an optimum value should be considered.

Based on the high-pressure apparatus' operable conditions, data gathered during the olive pomace oil supercritical extraction experiments, and data from a recent work [55] regarding enzymatic transesterification in supercritical carbon dioxide, the following parameters were selected for the current biodiesel production experiments:

- 40 MPa, 313 K
- 1:24 oil:methanol molar ratio
- CO_2 flow rates of 10 g/min and 15 g/min

For all experiments, approximately 45g of fine olive pomace – all from the finest residue fraction (<710µm) – and approximately 50g of enzyme were used. The CO_2 flow rate of 10 g/min was determined for a residence time of 11,6 mins, with the subsequent methanol content in the solvent being 1 wt.%. The CO_2 flow rate of 15 g/min was determined for a residence time of 7,33 mins, with the subsequent methanol content in the solvent being 1,1 wt.%.

3.4.2 Biodiesel Production Curves

For each reaction experiments, a curve of collected mass over time was traced. What follows is an example of such a curve.

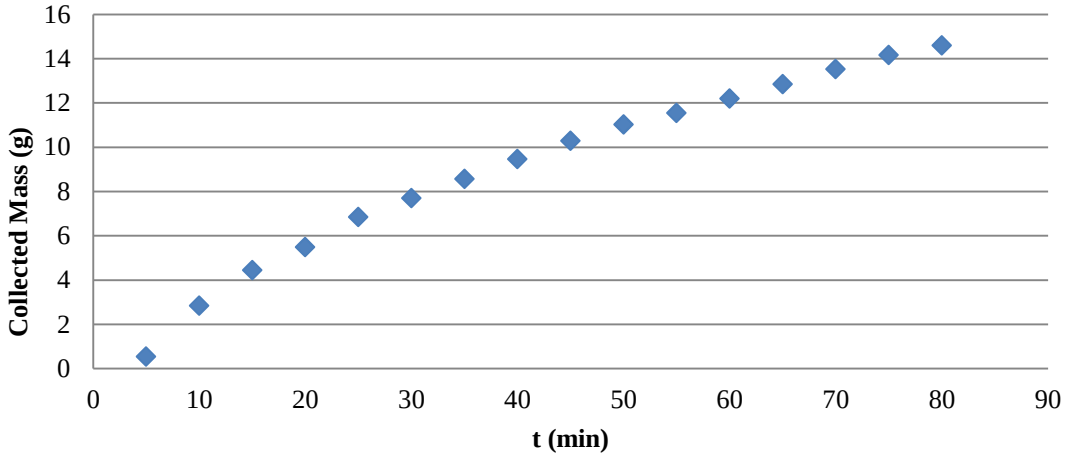


Figure 3.14 – Example of a biodiesel extraction curve – extracted mass (g) over time (min)

The mass never stabilizes over time because even after there is no more biodiesel to be collected, methanol continues to be injected into circulation unless stopped. For all experiments, the methanol was stopped at 65 minutes, and then $scCO_2$ was allowed to circulate for several minutes to wash the enzyme and the tubing of any remnants so that the enzyme can be reused for the next experiment.

The following figure shows the collected liquid common to all experiments. At first glance, a noteworthy difference was the yellow liquid compared to the green oil in the extraction experiments. It was also less viscous than the oil. This suggested that the triglycerides had been successfully transesterified, but this would only be confirmed with GC analysis of FAME content.

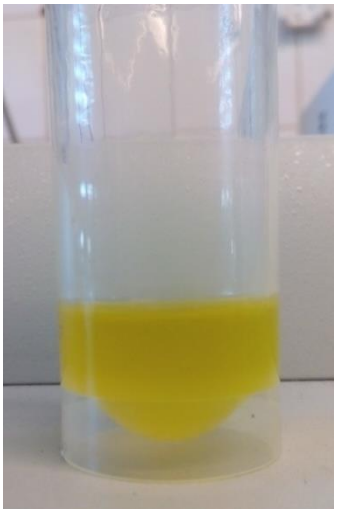


Figure 3.15 – The yellow liquid collected during the biodiesel production experiments.

No visible phase separation was observed for any sample. This suggests glycerol amounts are low, but they could not be accurately quantified due to unavailability of the analytical equipment for that.

3.4.3 Biodiesel Purity

The biodiesel purity of the collected samples, for each time interval, was analysed via GC, as described in Chapter 2.2.3.9.

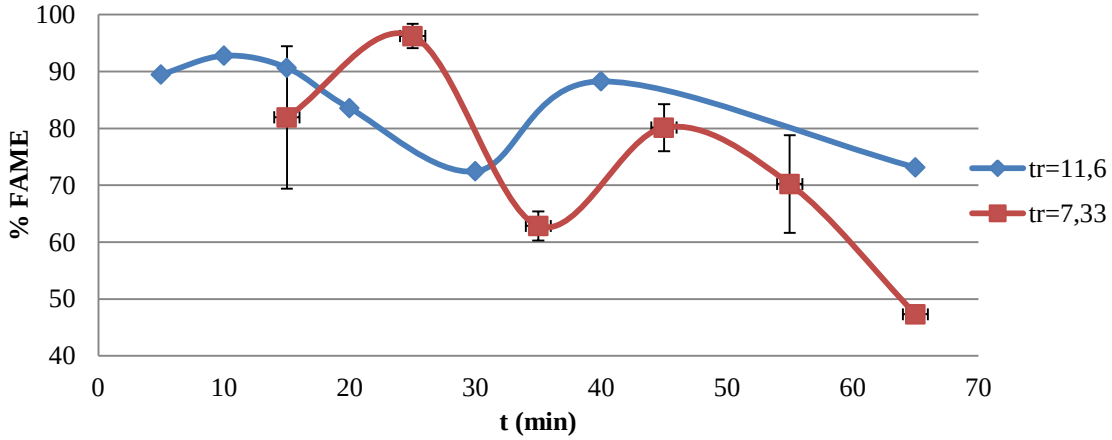


Figure 3.16 – Compilation of data regarding biodiesel purity in % FAME (m/m) over time

All reactions displayed an oscillation in terms of FAME purity over time, as shown in Figure 3.16. The error bars present in the figure for the t_r of 7,33 mins derives from the two experiments performed in those conditions, and represent the inconsistency of the FAME purity oscillation even when the experiments took place with the same conditions. It is possible that there was an accumulation of methanol in the reactor – due to the intentionally excessive molar ratio of methanol to oil – which resulted in the FAME being diluted in the collected liquid. This is possibly supported by the fact some of the collected samples having evaporated almost totally, leaving too little liquid for FAME testing – which means the sample had a very high methanol content. The higher FAME purity values at some time points are possibly due to periods of the experiment where methanol had not yet accumulated, or had temporarily been cleared from the reactor. The reaction with a t_r of 11,6 min had a reduced oscillation, possibly because there was a longer contact between the reagents and the enzyme, promoting a more consistent rate of reaction.

Towards the end of the reaction, a general decrease in the FAME purity can be observed, which is possibly due to a decrease in extractable oil content, on top of the methanol accumulation in the reactor. With the transesterification ceasing to occur, more methanol was gradually left unused for the reaction.

Nevertheless, a number of biodiesel samples with high FAME content were obtained, at a purity of 90% or more, which is in accordance with literature cases of biodiesel production from olive pomace oil with yields of 90% or more. [21,38,39,40] This happened despite the fact this is a proof of concept and not an experiment with thoroughly optimized parameters, demonstrating the potential of the integrated extraction/production/separation process for enzymatic production of biodiesel from olive pomace.

Due to the inconsistent FAME purity in the collected samples, it is not possible to assess if the lipase used maintained or not its activity across all experiments, even with the enzyme having been washed with $scCO_2$ after each experiment for reuse.

During GC analysis where the presence of FAME was confirmed, a FAME profile equivalent to the Fatty Acid Profile shown in Figure 3.13 was also observed, with methyl oleate being the most abundant FAME. This indicates that all fatty acids reacted in equal measure, as expected. The biodiesel obtained also had a linolenic acid methyl ester content lower than 1 wt.% across all samples, conforming to the EN14214 norm.

3.5 Phenolic Content of Olive Pomace

Finally, the last objective of this work to be discussed was the analysis of the phenolic content in olive pomace. As described in Chapter 1.10.1.1, olive pomace can contain a variety of phenolic compounds, serving as metabolites that can oxidize easily, granting them excellent antioxidant potential. Among the possible compounds, emphasis is given to hydroxytyrosol, but others can exist that have potential health benefits on their own, like oleuropein and tyrosol.

Due to the interest in this type of compounds and in the possibility for extraction of them from the olive pomace, this work included studies of $scCO_2$ based extractions of such compounds. At the same time the extracted oil samples were studied for their fatty acid profile and unsaponifiable matter content, they were also analysed for their phenolic content. The results of those studies were compared to conventional lab scale analysis of the olive pomace's phenolic content.

The procedures to extract and the phenols for analysis are described in Chapters 2.2.2.2 and 2.2.2.3 for the olive pomace and the $scCO_2$-extracted oils, respectively. Two types of analysis were made: total quantification of all phenols and individual quantification of each identifiable phenol.

3.5.1 Total Phenolic Content Quantification via Folin-Ciocalteu Method

As described in Chapter 2.2.3.6, the Folin-Ciocalteu method for total phenolic quantification was implemented for all extracted phenol samples. First, the total phenolic content of oven-dried and freeze-dried olive pomace were compared, to assess the effect of the heat involved in the oven-drying process on the phenolic content of the residue due to its heat sensitivity.

Table 3.5 – Total phenolic content of oven-dried and freeze-dried olive pomace.

Drying method	mg/kg
Oven-dried	452,94 +/- 69,44
Freeze-dried	502,65 +/- 59,52

Analysing the results displayed in Table 3.5, the freeze-dried olive pomace had a higher phenolic content than the oven-dried residue. This is not unexpected, since the freeze-drying process is less aggressive towards this type of compounds than the use of an oven. Even so, 90,1 +/- 3,6 % of the phenolic content was preserved in the oven-drying process.

The error intervals observed in Table 3.5 are possibly related to a lack of homogeneity of the olive pomace and to operator errors in the drying of each batch of alperujo. It should also be noted that even the maximum approximate values of 560 mg phenols/kg alperujo fall short of the literature interval of 6200-23900 mg phenols/kg alperujo. [34]

Following are the results for the total phenolic quantification for the analysed $scCO_2$ extracted olive pomace oil samples, along with the operating pressure and temperature conditions employed during the oil extraction. The results are displayed in mg phenol/kg pomace for comparison purposes with the results in Table 3.5.

Table 3.6 - Total phenolic content of scCO2 extracted olive pomace oil samples.

P/T conditions	mg phenol/kg pomace
50 MPa, 323 K	190,49 +/- 5,74
50 MPa, 348 K	282,92+/- 4,68
60 MPa, 323 K	282,26 +/- 25,06

It can be observed in Table 3.6 that greater phenolic concentrations are achieved when greater operating pressures and temperatures were employed in their respective oil extractions; much like oil extraction was optimized the same way. It can be concluded that the operating conditions of 60 MPa and 323 K led to the highest phenolic content in the oil. It is possible that the oil itself aided in the phenol extraction, acting as a non-polar co-solvent – the phenol extraction would then be performed not by carbon dioxide, but the combined oil/$scCO_2$ mixture.

Analysing both Table 3.5 and Table 3.6, it be determined that approximately 42 +/- 1,3 % of the phenolic content was extracted using the supercritical process at 50 MPa and 323 K, compared to the extracted amounts for the oven-dried pomace. For the $scCO_2$ extraction at 50 MPa and 348 K, the yield was 62,5 +/- 1 %, and for the $scCO_2$ extraction at 60 MPa and 323 K, the yield was 62,3 +/- 5,53%. Such a difference between oil and residue phenolic content is not

unexpected, since the methods involved in the isolation of phenols for quantification are different for both sample types.

3.5.2 Phenolic Content Determination via HPLC

As per described in Chapter 2.2.3.7, the phenolic extracts from both samples types – olive pomace and $scCO_2$ extracted olive pomace oil were analysed using a HPLC apparatus. Specifically, samples of freeze-dried olive pomace and of oil extracted using 60 MPa and 323 K as operating conditions were analysed, because they have been determined as being the pomace and oil samples (respectively) with the highest phenolic content.

The following table shows the determined concentrations for each individual phenol that was identified in this work, for each sample type.

Table 3.7 – Phenolic compounds identified and quantified on olive pomace derived samples

Phenolic Compound	**Concentration (mg/kg residue)**	
	Freeze-dried Olive Pomace	**$ScCO_2$ Extracted OPO (60 MPa, 323 K)**
Hydroxytyrosol	39,45 +/- 19,15	6,42[a]
Tyrosol	146,11 +/-7,04	49,37 +/-0,57
Oleuropein	32,18	24,76 +/- 16,48
Quercetin	15,46 +/- 0,73	2,03 +/- 0,12
Caffeic Acid	25,90 +/- 0,01	0
Ferulic Acid	2,5[a]	0
p-Coumaric Acid	12,75 +/-0,47	6,42[a]
Total Determined Content	274,35 +/- 27,4	89 +/-17,17

[a] The concentration was lower than the concentration gap that the standard curve covered (1ppm on the analysed extract, with equivalent concentration in the residue in the table)

Out of the sixteen possible phenols in olive pomace (as shown in Chapter 1.10.1.1), seven were identified – hydroxytyrosol, tyrosol, oleuropein, quercetin, caffeic acid, ferulic acid and p-coumaric acid.

For the hydro-alcoholic method derived samples, tyrosol was the most abundant phenol detected, followed by oleuropein and hydroxytyrosol. Hydroxytyrosol in particular was quantified with an error margin greater than the other compounds and oleuropein was only detected in one out of several attempts of detecting it, which could possibly be due to a non-homogeneous distribution of the compounds in the olive pomace. The least concentrated

compound was ferulic acid, in a concentration below its standard curve's minimum concentration value.

For the $scCO_2$ extracted oil samples, tyrosol was again the most abundant phenol, but hydroxytyrosol's concentration was below the standard curve's minimum concentration value, with the same happening for p-coumaric acid. Caffeic acid and ferulic acid were not detected in the oils, and oleuropein was determined with an error margin larger than the other compounds. Like hydroxytyrosol in the pomace samples, this could possibly be due not just to a non-homogeneous distribution on the pomace, but also due to inconsistent degradation of the compound during the oven-drying process.

All of the detected compounds existed in lower concentrations in the oil samples than the pomace samples, with the total quantified content of the seven detected phenols in the oil being approximately 33,1 +/- 9,9 % of the same content for freeze-dried pomace. This was expected since it was already determined in Chapter 3.6.1 that the total phenolic content in the oils was lower than in the pomace samples, and that the oven-dried pomace itself (from which the oils were extracted) already had less total phenolic content than the better-preserved content in the freeze-dried pomace. The lower concentrations of hydroxytyrosol and p-coumaric acid, and the absence of caffeic acid in the oil compared to the pomace could also possibly be due to a stricter selectivity of the $scCO_2$/oil mixture for phenol extraction due to their non-polarity.

CHAPTER 4: CONCLUSIONS AND FUTURE WORK

It was concluded that $scCO_2$ can extract oil from olive pomace at an efficiency of over 80% compared to a hexane based Soxhlet method, and that oil is more abundant in the finer pulp fraction of the pomace than in the seed fraction. Thus, a thorough homogenization procedure should be implemented to olive pomace before extraction procedures – either separation of the seeds from the pulp, or a more uniform grinding of the pomace to more uniform particle diameters. It was also concluded that pressure and temperature have an influence on the $scCO_2$ extractions, and based on optimized operating conditions of 50 MPa and 348 K and a fine pulp fraction of the pomace, an oil loading of 32,60 g oil/kg CO_2 was calculated.

Regarding biodiesel production, it was concluded that the transesterification of olive pomace oil to biodiesel, via combination of a lipase as a catalyst and supercritical carbon dioxide as a solvent, and combining the extraction of oil and its conversion to biodiesel in a single integrated process, was demonstrated as a proof of concept, with FAME purities of 90% being achieved. Nevertheless, the process is not optimized, and future work should include experiments with other residence times, other oil:methanol molar ratios, and other temperatures and pressure operating conditions in an attempt to optimize biodiesel production and achieve a more stable FAME purity over reaction time. There should then be more exhaustive studies of biodiesel properties to determine how well the biodiesel can conform to EN14124 norms.

Finally, regarding the olive pomace phenolic content, it was concluded that the oven-drying of the pomace has an influence on the pomace's phenolic content, with approximately 10% of the total phenolic content being lost. It was also concluded that the $scCO_2$ extraction has a lower efficiency compared to hydro-alcoholic extraction, with the olive pomace oil, even in the most optimized operating conditions, (60 MPa, 323 K), contained at most approximately 70% of the oven-dried pomace's total phenolic content. This was in accordance with the HPLC analysis of the seven compounds that were identified, in which it was concluded that $scCO_2$ extracted oils possessing less than half of the phenolic content equivalent to the seven detected phenols, and some of the phenols like hydroxytyrosol existing in the oil on a lesser order of magnitude compared to the pomace. A similar analysis to individual phenols on oven-dried pomace should be performed to assess how much of the difference is related to differences between the pomace drying processes, differences between $scCO_2$ based extraction and the hydro-alcoholic extraction method or a lack of pomace's homogeneity.

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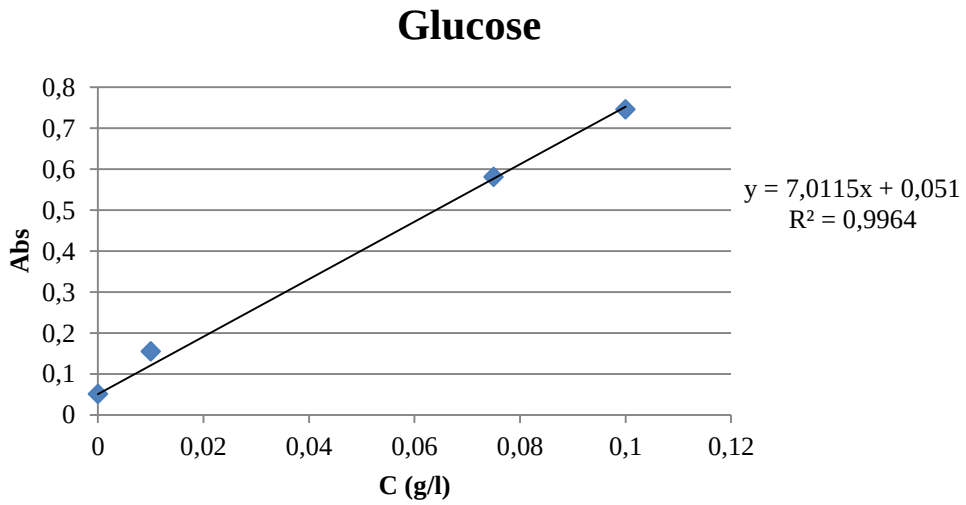
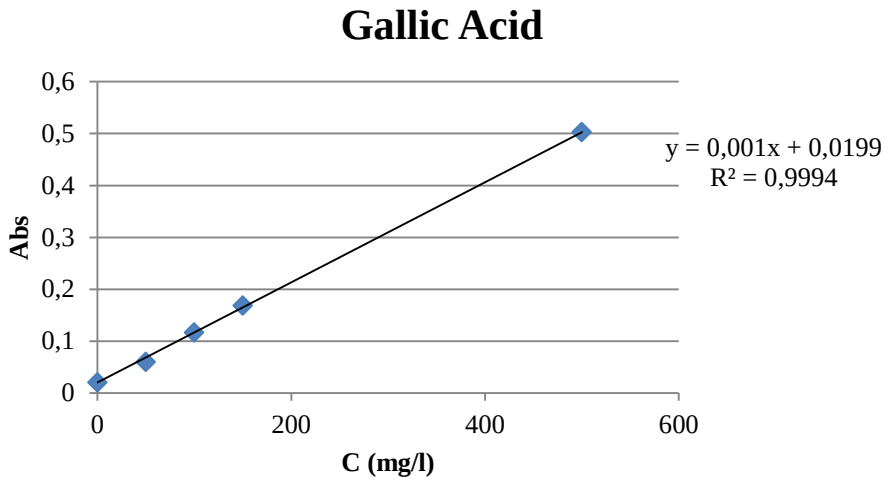
APPENDIX

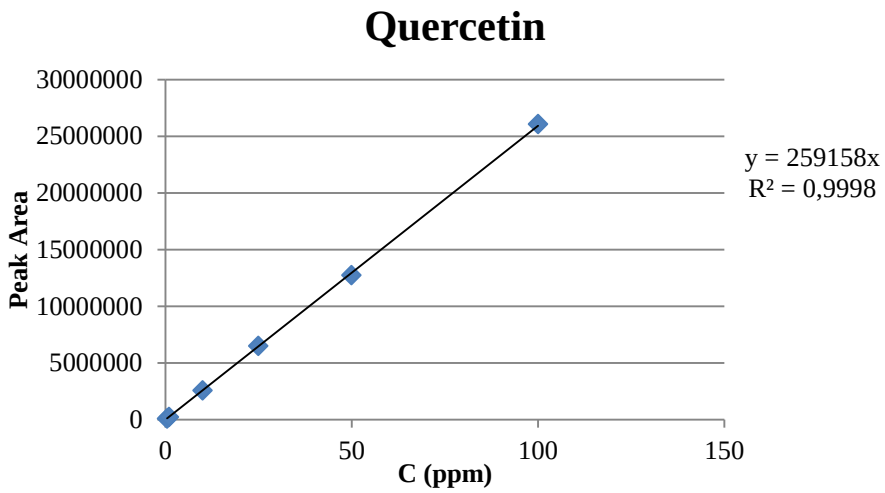
Appendix A – Standard for the Phenol-Sulphuric Method

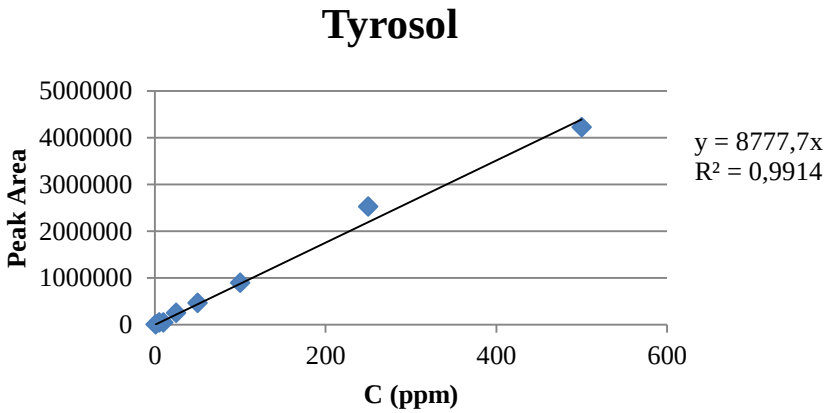


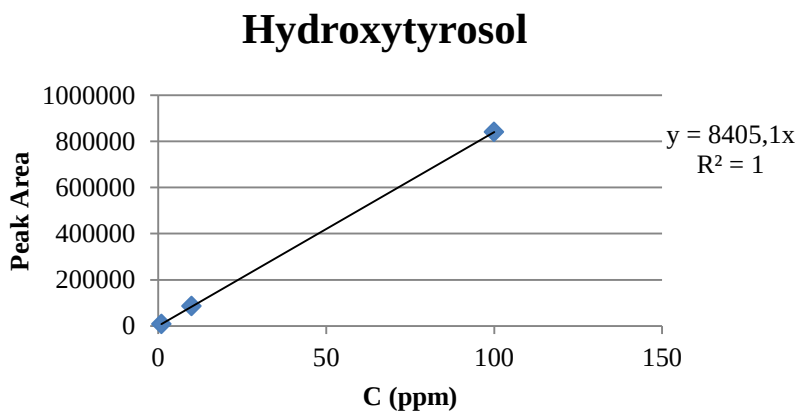
Appendix B – Standard for the Folin-Ciocalteu Method



Appendix C – Standards for HPLC Phenolic Compound Identification






Hydroxytyrosol
Peak Area
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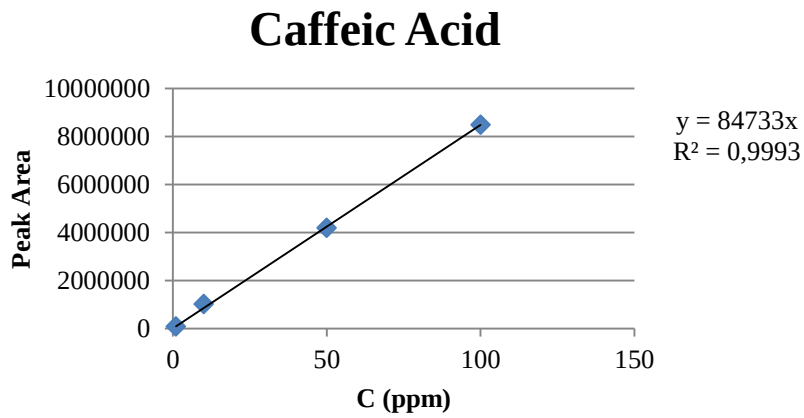
800000
600000
400000
200000
0
0
50
100
150
C (ppm)
y = 8405,1x
R² = 1


Caffeic Acid
Peak Area
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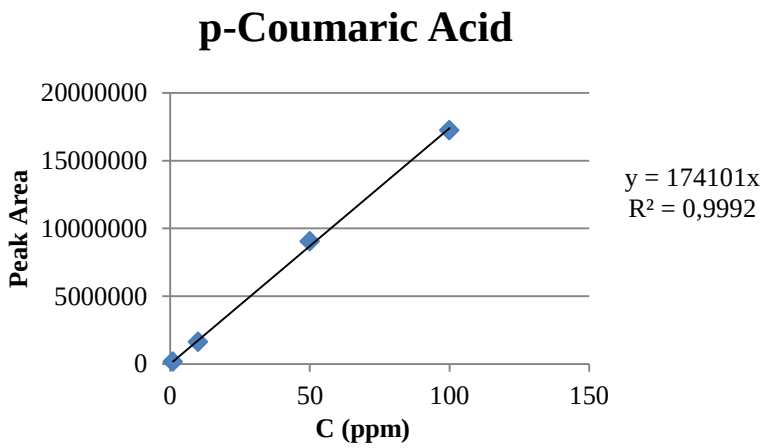
6000000
4000000
2000000
0
0
50
100
150
C (ppm)
y = 84733x
R² = 0,9993


p-Coumaric Acid
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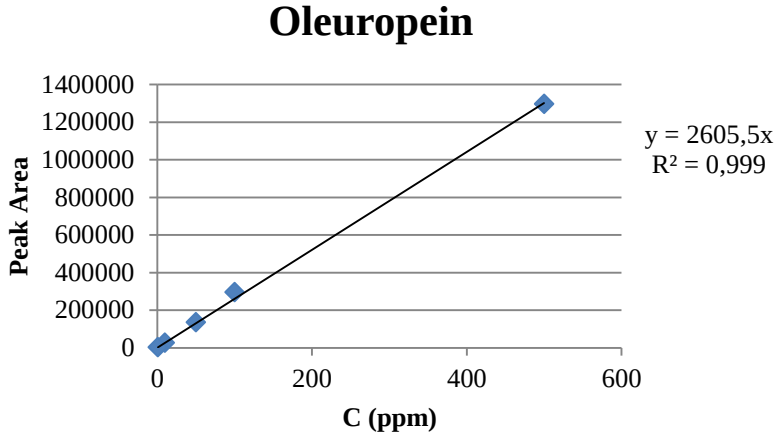
15000000
10000000
5000000
0
0
50
100
150
C (ppm)
y = 174101x
R² = 0,9992


Oleuropein
Peak Area
1400000
1200000
1000000
800000
600000
400000
200000
0
0
200
400
600
C (ppm)
y = 2605,5x
R² = 0,999